D515
T8

Vol. VIII, No. 6 December 1914

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

DR E. F. ARMSTRONG
PROF. V. H. BLACKMAN
PROF. A. J. BROWN
MR J. A. GARDNER
DR F. G. HOPKINS
PROF. F. KEEBLE
PROF. B. MOORE
DR W. RAMSDEN
DR E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C.
EDINBURGH: 100, Princes Street

also

H. K. LEWIS, 136, Gower Street, London, W.C.
WILLIAM WESLEY & SON, 28, Essex Street, London, W.C.
CHICAGO: The University of Chicago Press
BOMBAY AND CALCUTTA: Macmillan and Co., Ltd.
TORONTO: J. M. Dent & Sons, Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1914

Price Seven Shillings net.

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 25*s*., which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr R. H. Plimmer, University College, London, W.C., to whom subscriptions should be sent.

Papers for publication should be sent to Prof. W. M. Bayliss, F.R.S., University College, W.C., or to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£1. 1*s*. net per volume (post free) payable in advance) should be addressed to Mr C. F. Clay, Cambridge University Press, Fetter Lane, London, E.C.

The Journal is issued every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

The Cambridge University Press has appointed the University of Chicago Press agents for the sale of the *Biochemical Journal* in the United States of America and has authorised them to fix the following subscription price. $6.25 net.

Notice to Contributors.

1. Contributors receive 50 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

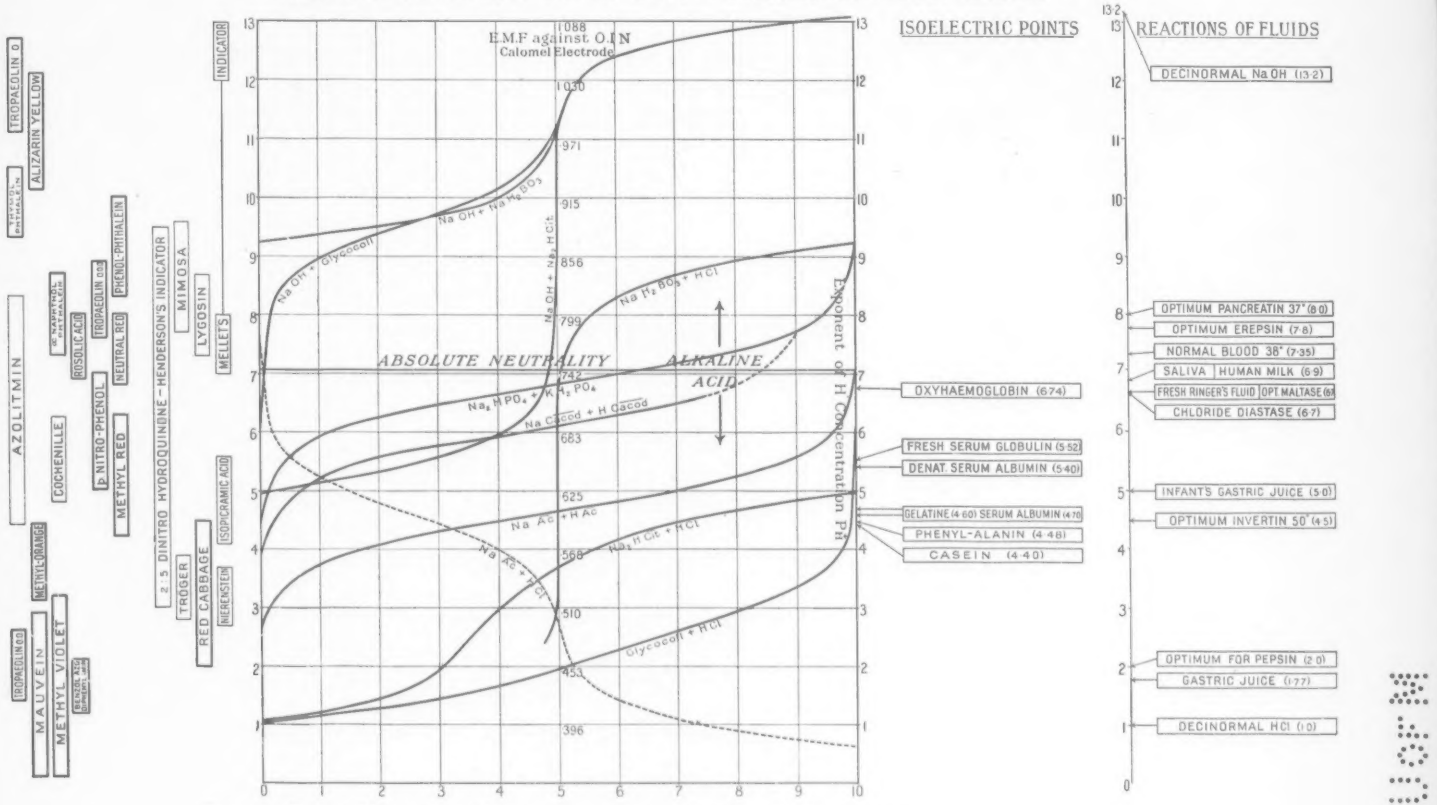
3. All communications intended for publication must be type-written.

4. References. References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912, 1), *Zeitsch. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus (Ackermann, 1912, 1).

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

CHART OF HYDROGEN ION CONCENTRATION DATA



PARTS IN TEN OF MIXTURE OF "MORE ALKALINE" OR "LESS ACID" CONSTITUENT
except in the case of the dotted curve ($NaAc + HCl$).

The Sörensen solutions are: 0·10N HCl; 0·10N NaOH; 7·505 g. glycocoll + 5·85 g. NaCl per litre; 11·876 g. $Na_2HPO_4 . 2H_2O$ per litre; 9·078 g. KH_2PO_4 per litre; 21·008 g. citric acid in 1 litre of 0·20N NaOH; 12·404 g. boric acid in 1 litre 0·10N NaOH. The other solutions are: 0·20N sodium acetate, acetic acid, sodium cacodylate, cacodylic acid. For details of plotting of dotted acetate-HCl curve, see notes.

Published by the Cambridge University Press, Fetter Lane, London
May be obtained from
Messrs BAIRD and TATLOCK, 14, Cross Street, Hatton Garden, E.C.
Price, 6d *net; post free*, 7d.

THE BIOCHEMICAL JOURNAL, VOL. VIII. NO. 6 (Walpole)

LVIII. THE ACTION OF POISONS ON REDUCTASE AND ATTEMPTS TO ISOLATE THIS ENZYME.

By DAVID FRASER HARRIS
and HENRY JERMAIN MAUDE CREIGHTON.

From Dalhousie University, Halifax, N.S., and Swarthmore College, Swarthmore, Pennsylvania, U.S.A.

(*Received Sept. 28th, 1914.*)

Although it has been shown [Harris, 1910] that tissue-reductase is comparatively insoluble, the following attempts at its extraction have been made; they led not to its being isolated but to a study of the action of poisons on it.

Extracts or solutions of reductase were made thus: 1. A liver extract; the livers of four pigeons, before the animal heat had left them, were crushed in the juice-press, ground up with sand in a mortar in the presence of 200 cc. of 0·75 per cent. sodium chloride solution and the mixture allowed to remain under toluene for 24 hours. 2. The pectoral muscles of two pigeons, still warm, were crushed in presence of glycerol and allowed to remain under this liquid for 24 hours. 3. The pectoral muscles of two other pigeons were crushed with glycerol and 0·75 per cent. sodium chloride solution and the mass allowed to stand for 24 hours. There were, then, three extracts or solutions of reductase; a saline extract of liver, a pure glycerol extract of muscle, and a glycerol and saline extract of muscle.

The presence of reductase in the extract was detected by determining whether it effected the reduction of the two-banded oxyhaemoglobin to the one-banded haemoglobin. In the reduction experiments, one part of extract was mixed with two parts of a blood solution prepared by diluting 10 cc. of freshly defibrinated cat's blood to 250 cc. with water. The reduction was carried out at 40°.

The progress of the reduction of oxyhaemoglobin to the fully reduced pigment was followed spectroscopically, a small direct vision spectroscope being employed. The replacing of the two bands of oxyhaemoglobin by the

one band of fully reduced haemoglobin gives an end-point which is not affected by any personal factor. But the disappearance of the two bands is from a very early period accompanied by the appearance of a fainter band between them in the intralinear green, so that at a certain stage of the reduction the single band of the reduced haemoglobin is flanked by the remains of the two bands of the oxyhaemoglobin. The pigment was never considered fully reduced until the remains of these margins of the single band had completely disappeared. In this way it was possible to obtain an end-point of considerable definiteness. It was evident that owing to the opacity of the mixture of extract and diluted blood in even a narrow test tube, the spectrum of the oxyhaemoglobin could not be seen sufficiently distinctly to be certain of the end-point. It was found that glass vessels made by drawing out short lengths of glass tubing (6 mm. diam.) to a conical end answered the purpose very well, for if we were in doubt as to the persistence of the two bands, when the spectroscope was directed across the tube itself, it was only necessary to look through the conical part of the tube where the mixture was sufficiently transparent to obtain a distinct spectrum. Complete reduction was considered to have taken place at the first moment the two bands had vanished, and when they were no longer distinguishable as ribbed margins to the single band.

It was previously ascertained that neither the glycerol, the saline solution nor a mixture of these effected the reduction of oxyhaemoglobin at 40°.

A mixture of the glycerol and saline extract of the pigeon's muscle with the blood solution had its oxyhaemoglobin completely reduced in two minutes in one experiment, and in three in another. Indeed the small quantity of residual blood in this extract of muscle was found to be in the reduced condition. The pure glycerol extract was incapable of effecting reduction. The saline extract of the liver completely reduced the oxyhaemoglobin in 16 minutes. There was evidence, therefore, that a certain amount of reductase passed into solution in a menstruum of glycerol and normal saline both from disintegrated liver and from muscle, but that evidently it did not pass into solution in the pure glycerol.

Efforts were next made to precipitate the reductase from these extracts or solutions. A quantity of the saline glycerol solution of hepatic reductase was thrown into an excess of absolute alcohol, and the precipitate allowed to stand for some time. The alcohol was then decanted, the precipitate washed with water and dried in a current of air at room temperature. This precipitate was shaken with glycerol and normal saline and some of the

resulting cloudy liquid mixed with dilute blood in a conical observation tube. No appreciable reduction could be detected at 40° even at the end of an hour. The filtrate from the alcohol precipitate had no reducing power. The toxicity of alcohol towards reductase was thus once more demonstrated, it having been previously found [Harris, 1911] that alcohol destroyed the power of reductase to reduce soluble Prussian blue. The glycerol and saline extract of the pectoral muscles was saturated with ammonium sulphate, but it was found that neither the precipitate itself nor the filtrate from it was able to effect any appreciable reduction of oxyhaemoglobin. In other words, ammonium sulphate inactivates or poisons reductase.

The poisonous influence of many substances towards enzymes has long been recognised, and has been the subject of considerable investigation. For example, the poisonous action of a number of substances on the catalase of the blood has been investigated by Senter [1903]. Indeed, many of the enzymic poisons are the same substances which inhibit the action of certain inorganic catalysts, as has been shown by the investigations of Bredig and his pupils during the last few years. The effect of different poisons on inorganic catalysts and enzymes is illustrated in the accompanying table, in which is shown the concentration of different poisons that is necessary entirely to destroy the catalytic action of colloidal platinum [Bredig and v. Berneck, 1899] and of catalase [Senter, 1903] on hydrogen peroxide.

TABLE I.

Poison	Colloidal platinum	Catalase
H_2S	1: 300000 molar	1: 1000000 molar
HCN	1: 20000000 „	1: 1000000 „
$HgCl_2$	1: 2000000 „	1: 2000000 „
$Hg(CN)_2$	1: 200000 „	1: 300 „
I in KI	1: 5000000 „	1: 50000 „
$NH_2(OH)HCl$	1: 25000 „	1: 80000 „
Aniline	1: 5000 „	1: 40000 „
As_2O_3	1: 50 „	1: 2000 „
CO	very poisonous	no paralysis
HCl	1: 3000 molar	1: 100000 molar
NH_4Cl	1: 2000 „	1: 1000 „
HNO_3	no paralysis	1: 250000 „

As our previous work has shown [Harris and Creighton, 1912] that the liver and other organs contain a reducing enzyme, reductase, and that attempts to isolate this enzyme have indicated that its activity is greatly reduced by contact with foreign substances, it seemed desirable to investigate the action of small quantities of a number of substances towards it. For

this purpose the reductase contained in cat's liver-juice was employed. The action of the foreign substances on the reductase was measured by determining the difference between the time required for the reduction of oxyhaemoglobin to the one-banded condition, by pure liver-juice, and by liver-juice containing some of the foreign substance with which it had been in contact for a definite time. The progress of the reduction was followed spectroscopically. In the experiments, a mixture of 1 cc. of fresh liver-juice and 1 cc. of an aqueous solution of the poison was allowed to remain in contact for ten minutes, at the end of which time 2 cc. of the blood solution (dilution 1 : 25) were added and the mixture introduced into observing tubes which were then placed in a thermostat at 40°. The spectrum of these mixtures was examined every half-minute. The times required for the reduction at 40° by liver-juice which contained foreign substances were compared with the time required for the reduction, at the same temperature, of a *normal* mixture containing 1 cc. of liver-juice, 1 cc. of distilled water and 2 cc. of the blood solution. Only such substances were employed as were found to produce no change in the spectrum of oxyhaemoglobin. It was not possible, therefore, to use a number of desirable substances such as copper sulphate, iodine, acids, alkalies, etc. The results of the experiments are given in Table II. The times given in this table represent the means of several experiments which seldom differed by more than a few per cent. and which, in most cases, were identical.

TABLE II.

Ten minutes required for reduction of normal mixture.

	Time required for the reduction of mixtures containing 1 cc. of liver-juice, 2 cc. of blood solution and 1 cc. of an aqueous solution of the poison having a concentration of	
Poison	0·01 molar	0·1 molar
As_2O_3	33 minutes	
KCN	30 „	34 minutes
$HgCl_2$	17 „	blood destroyed
Na_2AsO_3	17 „	„ „
$AuCl_3$	15 „	16 minutes
OsO_4	13 „	19 „
$MnCl_2$	11 „	25 „
NH_4Br	11 „	13 „
HCHO	10 „	48 „
NH_4Cl	9 „	9 „

The solution of As_2O_3 employed was somewhat more dilute than 0·01 molar.

In Table II the different substances are arranged in the order of their poisoning action with respect to the 0·01 molar concentration. The order is quite different with respect to the higher concentration. At the higher concentration some of the substances are relatively much more poisonous than at the lower. Thus formaldehyde, which exhibits no toxic action in the smaller concentration, is the most powerful poison in the list when its concentration is increased to 0·1 molar.

The poisonous action of the foregoing foreign substances is of physiological interest. A new method of studying the action of poisons on one phase of tissue-respiration has been used. It is possible that it may be found a convenient one where injection of poison into the entire animal is not desirable. One substance only of those examined exerted no retarding action on reductase, namely, ammonium chloride. This is in accordance with what is known of ammonium chloride therapeutically; it is a mild expectorant and a ciliary excitant and is not a toxic substance at all in the ordinary meaning of the word. It may be said that the more poisonous a substance is, the longer is the time required for reductase to effect complete reduction in the presence of the weaker concentration of the substance. Judged by this, neither ammonium bromide nor manganese chloride is a poison, and this, at least as regards the former salt, is quite in agreement with the experience of therapeutics. Ammonium bromide, except in exceedingly high doses, is not a poison. It is, however, exceedingly interesting that mercuric chloride and potassium cyanide, for instance, substances well known to be most energetic in destroying the activity of the inorganic ferment colloidal platinum and of catalase should be among the most toxic of the substances which were examined. In concentrations so dilute as 0·01 molar they interfered with the activity of reductase, causing the reduction to be lengthened one and a half times in one case and three times in the other. Quite distinctly toxic, even in such dilution, is sodium arsenite. Not far behind it come gold chloride and osmic acid, the latter a well-known protoplasmic poison. It need be a matter of no surprise that eight of the ten substances examined, more or less at random, should be toxic for reductase. None of these substances is a constituent of the normal cell-lymph in which reductase must act. The slightest trace of any of these salts or acids is foreign to the environment of living cells, and as such cannot but be highly injurious to them. Nevertheless reductase is found within limits to be fairly resistant, for concentrations of these poisons ten times as high as 0·1 molar did not entirely prevent it exerting its reducing action. In the case of formaldehyde

the time taken to reduce at the higher concentration, 0·1 molar, was more than quadrupled, being prolonged to 48 minutes.

That dilute formaldehyde should not exert a poisonous action on tissues is in keeping with what is believed to be its rôle in the anabolic processes carried out by vegetable protoplasm. Formaldehyde has been proved to be the first organic substance produced in the synthesis of carbohydrate in the plant, under the influence of sunlight on chlorophyll. This function of formaldehyde is incompatible with its being in high dilutions a protoplasmic or enzymic poison.

The fact that the poisons studied behave towards some substance in fresh tissue exactly as they behave towards catalysts in general, is an additional indication that that substance in tissue-juice is itself catalytic.

The physiological significance of the relative insolubility of reductase is undoubtedly this, that its sphere of action is *within* the cell. Inside the cell but separated from the oxygen-containing lymph by only the more or less permeable membrane or wall of the cell, the reducing enzyme extracts oxygen from the circumambient lymph. Reductase could not perform this particular service in tissue-respiration if it could dissociate from cell proteins and become dissolved in the lymph and blood. It would then circulate and perform a rôle wholly different from that which it evidently does perform at the surface of, but still inside, the living cells. Reductase is not a secretion, it is an *endo*-enzyme.

Summary.

1. It has been found that the reductase contained in pigeon's liver and muscle is soluble to some extent in normal saline, and in a mixture of normal saline and glycerol but not in pure glycerol. All attempts to isolate the active enzyme have failed.

2. The poisonous action of ten substances, chosen more or less at random, towards reductase has been investigated.

3. In conclusion, the action of the poisons on reductase and the insolubility of the latter have been discussed from the physiological standpoint.

References.

Bredig, G., and Muller v. Berneck (1899). *Zeitsch. physikal. Chem.*, **31**, 258.
Harris, D. Fraser (1910). *Biochem. J.*, **5**, 143.
—— (1911). *Biochem. J.*, **6**, 151.
—— and Creighton, H. J. M. (1912). *Proc. Roy. Soc.*, B. **85**, 487.
Senter, G. (1903). *Zeitsch. physikal. Chem.*, **44**, 257.

LIX. ON THE RESPIRATORY EXCHANGE IN FRESH-WATER FISH. Part II: ON BROWN TROUT.

By JOHN ADDYMAN GARDNER and CONSTANCE LEETHAM.

From the Physiological Laboratory, University of London, S. Kensington.

(*Received Nov. 5th, 1914.*)

The influence of diminution of oxygen content of water on fish life has been investigated by many observers, notably by Duncan and Hoppe-Seyler [1893], Chopin and Nikitin [see Kupzis, 1901] and König and Hünnemeier [1901]. The results generally indicate that fish—of very different varieties, including trout—die when the oxygen content of the water reaches 1–0·4 cc. per litre.

In the method employed by most observers the fish were placed in a closed vessel, and the gradual diminution of the amount of dissolved oxygen was brought about by the breathing of the animals themselves. When asphyxia took place samples of the water were withdrawn and analysed.

The subject has been more recently dealt with by Hans Winterstein [1908], who made use of roach (*Leuciscus erthyrophthalmus*). In his experiments the tension of the dissolved oxygen was reduced by passing through the water a continuous stream of nitrogen, and his method was not very dissimilar from that adopted in the experiments to be described in this paper.

Winterstein found that in the case of roach an oxygen content of 0·7 cc. per litre, or a tension of 2·2 % of an atmosphere, was compatible with life, but when the oxygen was reduced to 0·4–0·5 cc. per litre, *i.e.* a tension of 1·3–1·5 % of an atmosphere, asphyxia took place.

In Part I of this series of papers [1914], we showed that trout use more oxygen per kilo. per hour than some other varieties of fresh-water fish, and further that within the limits of temperature of our climate the oxygen absorbed was a function of temperature. Between 10° and 20° C. the oxygen absorption per kilo. per hour was approximately doubled. As the volume

of water that must be pumped through the gills is inversely as the oxygen content of the water, it seemed likely that the asphyxial tension would be greater at higher temperatures than at lower, and this difference would be dependent on the facts that at the higher temperatures the fish require more oxygen, and that under such conditions more work has to be performed to obtain the oxygen. It seemed of interest therefore to investigate the effects of diminishing tension of oxygen in the case of trout, and to determine the least quantity of oxygen per litre compatible with life at various temperatures.

The method adopted was a modification of that described in our earlier paper. The animals were placed in an hermetically closed vessel containing known volumes of water and air. The air was displaced by a *continuous*

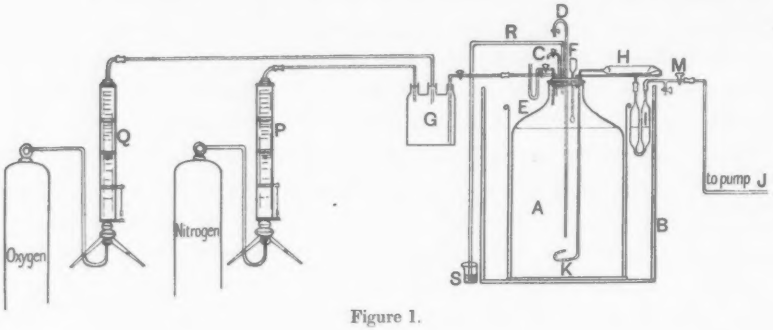


Figure 1.

current of nitrogen, or a mixture of nitrogen and oxygen of known composition. The gas above the water was then pumped through the water continuously. If the gas above the water consisted of nitrogen, the quantity of oxygen dissolved in the water was gradually reduced. Samples of water were withdrawn when the fish entered into the asphyxial condition, or at any desired point prior to this, and analysed. If the gas above consisted of any definite mixture of oxygen and nitrogen, the oxygen in the water was gradually reduced until equilibrium was attained between the gas above the water and that in solution, and the fish could then be observed under any desired oxygen tension.

Apparatus employed.

The apparatus used was that fully described in Part I with some modifications. The essential parts of the apparatus with the modifications introduced

are shown in Figure 1, but the pumping mechanism is omitted, as it was exactly the same as in the earlier experiments.

In these experiments a much larger volume of water was used—about 45 litres—and the gas space above the water was consequently only 2–3 litres in capacity. Only one fish of about eight inches in length and approximately 90–100 grams in weight was used in each experiment. The reason for using only one animal and a relatively very large volume of water was to prevent any appreciable increase in the carbon dioxide pressure during the experiment. The oxygen reservoir in the old apparatus was replaced by a small mixing bottle *G*, of about 0·5 litre capacity, connected through two gas measuring instruments *P* and *Q* with cylinders containing respectively compressed oxygen and nitrogen. In this way pure nitrogen or any desired mixture of oxygen and nitrogen could be delivered continuously into the gas space above the water in *A*.

The excess of gas escaped through the tube *R* which was just closed by a water seal *S*. The meters *P* and *Q* were "Rotameters" made by the Deutsche Rotawerke Co. of Aachen. The "Rotameter" consists of a glass tube slightly increasing in diameter from below upwards and divided in a "litres-per-hour" scale. The gas on entering from the lower opening sets in rapid rotation, about a vertical axis, a cylindrical float on the side surface of which a spiral groove is cut. The float rotates freely and visibly in the gas stream without coming in contact with the walls of the tube, and rises in the tube to a height corresponding to the volume of the gas passing through. The tube is marked with an empirically graduated scale giving the litres per hour. The oxygen meter was graduated to read from 0 to 50 litres per hour, and the nitrogen meter from 0 to 100 litres. Analyses of the mixed gases showed that the instruments gave reasonably accurate results—quite as accurate as would be obtained by the use of any of the ordinary forms of volume meters—and they were much more convenient. In performing an experiment the bottle *A* was filled to the desired point with tap water and left in the thermostat *B* to attain the temperature required. A weighed fish was then introduced; the air in the gas space was displaced by nitrogen or any desired mixture of oxygen and nitrogen, and a current of this gas passed through at a known rate. The gas in the space above the water was then pumped through and through the water by means of the pumping mechanism *I*, *H*, and spray *K* at a rate of about 100 cc. per few seconds. The rate could be varied by adjustment of the pumping mechanism. In this way the composition of the gas dissolved in the water was

gradually changed to that corresponding to the partial pressure of the gases in the space above. This was a slow process, owing to the large bulk of water employed, and, even when pure nitrogen was passed through the gas space, it took several hours, the exact time depending on the rate and efficiency of spraying, to get rid of the oxygen in the water. Measured samples of water were withdrawn by displacement of mercury in the manner described in our former paper, at desired periods. The gases were pumped out of the samples by means of the pump described by Buckmaster and Gardner [1910, 1912] and analysed in the usual way. An example will make clear the mode of calculation. A fish weighing 80 g. was placed in the apparatus at 1 p.m., and the current of nitrogen started at 65 litres per hour. The temperature of the water was 6° C. The pump was now started and at 4.40 p.m. the fish turned over on his back in an asphyxial condition. A sample of water was now withdrawn, measuring 53·5 cc. This sample yielded to the pump gas which after standing over potash to get rid of carbon dioxide measured 1·83 cm. at 14·15° and constant volume (48·65 cc.). After treatment with alkaline pyrogallate it measured 1·76 cm. at 14·10°. The water therefore contained 0·79 cc. of oxygen and 20·02 cc. nitrogen, measured at 0° and 760 mm., per litre. Taking Winkler's value 0·04181 as the absorption coefficient of oxygen at 6° (Landolt, Börnstein, Meyerhoffer's tables) the partial pressure of the oxygen is $\frac{0{\cdot}79}{10 \times 0{\cdot}04181} = 1{\cdot}89$ per cent. of an atmosphere.

The results, we believe, are accurate. They do not depend, as in the experiments described in Part I, on analytical differences before and after a period of time, and are not therefore affected by the sources of error there described.

Experimental results.

It was easy, of course, to determine the gas content of the water at the point at which the animal entered into the asphyxial condition, but it was very difficult to find out at what point prior to this the animal began to suffer inconvenience.

Observations on the behaviour of trout under diminishing oxygen pressure showed that between 10° and 25° the animals exhibited strikingly similar behaviour. With fully saturated water the animals moved about continually in leisurely fashion, breathing in a normal manner. As the oxygen diminished the respiratory movements gradually increased in rapidity and force, and acquired a dyspnoeic character, as has been described by Baglioni [1908]

and other observers. At the beginning the animals spent the greater part of their time moving about gently. As the oxygen gradually diminished, they became more active, rushing about and jumping into the gas space above the water. These periods of activity alternated with periods of rest at the bottom with the dyspnoeic respiration noted. Gradually the periods of rest became longer relatively to the periods of activity, and ultimately the fish remained for the bulk of the time at the bottom with occasional short rushes to the surface. As the oxygen approached the asphyxial tension they remained all the time at the bottom and ultimately quietly rolled over on their backs. In this condition they died in the course of three or four minutes, but if at this stage oxygen was sprayed through the water they recovered in the course of 5 to 10 minutes, and afterwards appeared to suffer no apparent ill effects. The asphyxial condition evidently takes place within quite small limits of oxygen tension. At or below this they die, but slightly above they recover. Their behaviour can, we think, be explained by the increased volume of water the animals have to pump through their gills to get the necessary oxygen, as the tension of the gas diminishes below a certain value, necessitating the expenditure of a considerable amount of energy.

In quantitative experiments samples of water were withdrawn when the animals turned over on their backs and were in an asphyxial condition. Before reaching this point it was difficult to decide exactly when the animal began to suffer inconvenience, but an arbitrary point was selected, viz. when the animal's periods of rest at the bottom became well established, and the alternating periods of activity were quite short. The point arbitrarily selected was when the periods of rest were about three minutes, the active periods occupying only a few seconds.

At temperatures below 6° or thereabouts the animals behaved rather differently. At these low temperatures the animals, at the beginning, remained quietly at the bottom, with only occasional excursions round the bottle and to the surface. This continued for a considerable period and until the oxygen tension was very considerably reduced. No marked dyspnoeic respiration was noticed. As the oxygen tension further diminished the fish became very active, and ultimately spent most of their time rushing round near the surface, and often jumping and thrusting their noses out into the gas above the water. Asphyxiation generally took place during a period of activity and the fish fell over on their backs and gradually sank. Death took place rapidly, but again if oxygen was sprayed through recovery took

place in a very few minutes. At these low temperatures samples were taken at the asphyxial point only.

The results of a number of experiments are given in Table I.

TABLE I.

		Arbitrary initial pt.		Asphyxial point		Normal	
Weight of fish	Temp. of exp. °C.	c.c. of oxygen at 0° and 760 in water per litre	Partial pressure in % of 1 atm.	c.c. of oxygen at 0°, 760 in water per litre	Partial pressure % of 1 atm.	c.c. of oxygen 0°, 760 in water per litre	Partial pressure in % of 1 atm.
90 g.	6·4°	—	—	0·79	1·89	8·6	app. 21 %
90 „	9·5°–10°	3·75	9·86	0·81	2·19	8·0	
80 „	18°	3·19	9·9	1·49	4·62	6·61	
80 „	17°	5·05	15·4	1·37	4·17	6·75	
90 „	24°	3·55	12·33	1·97	6·83	5·89	
88 „	25°	3·18	11·23	2·4	8·49	5·78	
1	2	3	4	5	6	7	8

In a number of other experiments fish were kept for the greater part of a day at tensions which analysis showed were between the arbitrary points given in column 3 of the above table and the asphyxial points without any apparent subsequent ill effects, though judging by their dyspnoeic breathing the animals were in a state of distress. How long the animals could have existed in such a condition we did not attempt to determine. Winterstein kept roach under like conditions at a tension above the asphyxial point for about a week, but we did not push our experiments with trout to such lengths. Experience showed us that trout do not take kindly to being kept in a confined space for long periods, and to keep them healthy a very considerable space per fish is necessary, and it was felt that if conclusive results were to be obtained by keeping fish in the above apparatus for long periods a very large number of experiments would be necessary, and the fish would have to be observed after returning to the aquarium for long periods further. Our trout accommodation did not permit of this.

Discussion of results.

A glance at columns 5 and 6 of Table I will show that the oxygen content of the water when asphyxiation took place was much higher than was found by Winterstein in the case of roach. The oxygen content at 6°–10° was of much the same order as that at which he was able to keep roach alive for a week.

In our earlier paper we showed that the oxygen consumption of trout in fully saturated water increases with increase in temperature—within the ordinary limits of temperature it doubles itself for a rise of 10°,—and consequently the fish would have to pump a much larger volume of water through their gills at a higher temperature than at a lower to obtain the necessary amount of oxygen. Hence as pointed out at the beginning of this paper we should expect the oxygen content at the asphyxial point to be greater at higher temperatures than at lower. This conclusion is fully borne out by the figures in columns 5 and 6. The rate of increase with temperature of the figures in column 5 appears to be somewhat greater than the rate of increase in oxygen consumption under normal conditions of fully saturated water. This we think can be well explained by the increased work done in respiration under low tension of oxygen at higher temperatures. The figures in column 3 at the arbitrary point selected do not appear to show much variation, but we know nothing of the other factors governing them.

From a consideration of our own work and that of other observers there can be no doubt that trout can exist at tensions of oxygen considerably lower than normal, but judging by the type of respiration and the general behaviour of the animal, the trout under conditions between those represented in columns 3 and 5 were in a condition of distress which even in their natural environment would have a very deleterious effect. Such conditions of oxygen content might of course be readily produced by pollution of the natural water.

We take this opportunity of expressing our thanks to the Government Grants Committee of the Royal Society for aid in carrying out this work.

REFERENCES.

Baglioni (1908). *Zeitsch. allgem. Physiol.*, **7**, 177.
Buckmaster and Gardner (1910). *J. Physiol.*, **40**, 374.
—— (1912). *J. Physiol.*, **43**, 406.
Duncan and Hoppe-Seyler (1893). *Zeitsch. physiol. Chem.*, **27**, 165.
Gardner and Leetham (1914). *Biochem. J.*, **8**, 374.
Kupzis (1901). *Zeitsch. Nahr. Genussm.*, 631.
König and Hünnemeier (1901). *Zeitsch. Nahr. Genussm.*, 385.
Winterstein (1908). *Pflüger's Archiv*, **125**, 73.

LX. THE CHEMICAL INVESTIGATION OF THE PHOSPHOTUNGSTATE PRECIPITATE FROM RICE-POLISHINGS.

By JACK CECIL DRUMMOND and CASIMIR FUNK.

From the Department of Chemical Physiology of the Cancer Hospital Research Institute.

(*Received Nov. 5th, 1914.*)

The chemistry of rice-polishings has attracted many workers ever since it was shown that there occurred in the polishings a substance, apparently of a relatively simple chemical constitution, which had a powerful curative effect when administered in cases of beri-beri, or experimental beri-beri, produced by a diet of polished rice.

Practically all the communications which have appeared on the subject have, however, concerned themselves chiefly with that particular fraction of the polishings which contained the curative substance and have to a greater or less extent neglected other fractions which were obtained.

The objects of this investigation were, primarily, to repeat the attempt to isolate the curative substance in a pure condition, and, at the same time, to carry out as exhaustive an examination of the other simple nitrogenous substances contained in the phosphotungstic acid precipitate as possible, in the hope that some new substance might be isolated which could be regarded as a degradation product of the unstable curative substance.

We will not give a detailed account of the findings of previous workers, but will refer to and discuss them in the course of the description of our work.

Experimental.

For the purpose of this investigation 620 kilograms of rice-polishings[1] were rendered practically free from fatty substances by petroleum ether extraction, after which they were well extracted three times with alcohol in

[1] This fractionation was begun in 1913 [Funk, 1913, 1], but only the silver nitrate-baryta fraction was thus investigated.

a large extractor. The combined alcoholic extracts, which possessed a deep golden colour, were then freed from alcohol under diminished pressure at 30°, and yielded a thick, dark brown syrup.

This residue was now extracted with 10 per cent. sulphuric acid and the extract diluted with an equal volume of water. On the addition of a 50 per cent. solution of phosphotungstic acid a heavy precipitate was thrown down which was filtered off, washed with 5 per cent. sulphuric acid, and dried.

The dry phosphotungstates, which were to be investigated, were now decomposed by excess of baryta, and, after removal of the excess of baryta, the filtrate was neutralised with hydrochloric acid and concentrated to a thick syrup at low temperature and under diminished pressure.

Extraction of this syrup with alcohol removed a certain amount of inorganic salts, which remained behind insoluble and which for the most part consisted of potassium salts, and the alcoholic extract, after diluting with an equal volume of water, was precipitated by alcoholic mercuric chloride solution.

The heavy white sublimate fraction was filtered off, and, after removal of the excess of mercury from the filtrate and evaporation in vacuo, the purines present were precipitated together with silver chloride by the addition of 20 % silver nitrate in dilute nitric acid solution.

The combined precipitate of silver purine compounds and silver chloride was removed by filtration, and to the filtrate sufficient excess of silver nitrate solution was added to ensure that all compounds present which were capable of forming silver compounds had done so, when they were precipitated as such by the addition of an excess of baryta.

The filtrate from the silver nitrate-baryta fraction was now freed from excess of silver by means of hydrochloric acid, and from excess of baryta by sulphuric acid and the remaining basic substances were precipitated by the addition of phosphotungstic acid.

Four fractions were thus obtained:

1. Sublimate fraction;
2. Purine fraction;
3. Silver nitrate-baryta fraction;
4. Residual phosphotungstic acid fraction;

and these were investigated separately.

1. Investigation of the Sublimate Fraction.

This fraction, which weighed, when dry, 1505 g., was decomposed in aqueous suspension by means of sulphuretted hydrogen. The filtrate from the mercury sulphide possessed a bright blue colour which, however, rapidly faded on standing. It was decided to investigate this filtrate by two different methods, and accordingly it was divided into two equal portions.

Method I.

This half of the filtrate, in which the basic substances were present in the form of their hydrochlorides, was treated with silver nitrate and silver nitrate and baryta so as to obtain a purine fraction, a histidine fraction, and an arginine fraction. The final filtrate from these separations being freed from excess of baryta and silver was reprecipitated by phosphotungstic acid.

The four sub-fractions of the sublimate fraction were then investigated individually.

Purine sub-fraction of the sublimate fraction. This weighed 220 g. and consisted for the most part of silver chloride. It was ground up with water and the silver removed by sulphuretted hydrogen.

The filtrate from the silver sulphide, on concentration in vacuo at 30° C. to dryness, yielded a small, dark-coloured semi-crystalline residue. On recrystallisation from dilute alcohol, a very small amount of a slightly dirty white, granular substance was obtained. This compound on heating darkened at 220°, but did not show a melting point below 290°. Micro-analysis was attempted, using the method of Pregl, but gave variable figures, and it was found that the substance was not sufficiently pure for an elementary analysis to be made.

It was attempted to make a derivative which could be sufficiently purified to analyse, but the only one which could be isolated with readiness was the picrate, of which, however, we did not obtain sufficient to carry out an analysis.

The substance was a purine, gave a positive reaction with the xanthine test and the diazobenzenesulphonic acid test, a negative result with Wheeler and Johnson's reaction, and a doubtful positive reaction with Capranica's test for guanine.

The picrate was rather insoluble in cold water but more readily soluble in hot, insoluble in alcohol and showed no melting point below 290°.

From the bulk of the evidence we considered that we had isolated guanine, but in the absence of analytical data we could not be certain of this point.

The filtrate from the guanine picrate yielded about a milligram of a crystalline picrate melting without further purification at 145°, but owing to the extremely small amount at our disposal we were unable to make any further examination of this substance.

No other substances were isolated from this fraction.

Histidine sub-fraction of the sublimate fraction. A small fraction which weighed 9 g. It was decomposed in the usual manner in aqueous suspension by means of sulphuretted hydrogen, and the filtrate from the silver sulphide on concentration in vacuo at 30° yielded a very small amount of a residue which was obviously very impure.

This residue was taken up in 200 cc. of water and precipitated by alcoholic mercuric chloride. The white mercuric chloride salt was decomposed, and yielded on concentration of the filtrate from the mercury sulphide a small residue from which a crystalline platinum chloride salt was prepared.

The platinum double salt, after one recrystallisation from dilute alcohol, crystallised in fine needles, melted at 239–240°, and gave on analysis, after drying at 100° C. in vacuo, the figures:

7·643 mgms.; 2·439 mgms. platinum; 31·91 % Pt.
Calculated for choline platinichloride; 31·73 % Pt.

No substance could be isolated from this fraction other than this small amount of choline which was probably in this fraction as an impurity.

Arginine sub-fraction of the sublimate fraction. This fraction, which only weighed 0·4 g., did not give us a sufficient residue on decomposition to enable us to identify or isolate anything from it, other than a small trace of inorganic salts.

Phosphotungstic acid sub-fraction of the sublimate fraction. This final sub-fraction was decomposed by means of lead acetate and the filtrate from the lead phosphotungstate freed from lead, acidified with hydrochloric acid and concentrated at low temperature and under diminished pressure to a thick syrup. Extraction of this syrup with absolute alcohol left a small amount of insoluble inorganic salts, and the alcoholic extract was then precipitated with alcoholic mercury chloride.

The heavy sublimate salt was recrystallised twice from water and then decomposed. The filtrate from the mercury sulphide was concentrated as far as possible and the thick syrup thus obtained allowed to stand in an

evacuated desiccator over potash and calcium chloride. After some weeks there was obtained a crop of fine needle-shaped crystals which were extremely hygroscopic and closely resembled choline hydrochloride in appearance. A platinum double chloride was prepared, and, after one recrystallisation from dilute alcohol, melted at 240°.

Analysis (substance dried at 100° in vacuo).

22·155 mgms.; 7·023 mgms. platinum; 31·71 % Pt.
Calculated for choline platinichloride; 31·73 % Pt.

A fine crystalline picrate was also obtained, which on recrystallisation from water separated in fine clusters of golden yellow prisms which melted sharply at 240°.

The presence of comparatively large amounts of choline in rice-polishings has been noticed by many previous workers [Funk, 1911; Suzuki, Shimamura and Odake, 1912; etc.].

The mother liquors from the recrystallisation of the choline mercury chloride were decomposed by means of sulphuretted hydrogen and the filtrate from the mercury sulphide concentrated at low pressure and temperature to a thick syrup. Extraction of this syrup with cold alcohol yielded a substance which was rather insoluble in alcohol and which was not inorganic in nature. It was removed by filtration and after washing with alcohol examined. Without further purification it melted at 242° and gave a fine double salt with platinic chloride.

This salt, after one recrystallisation from dilute alcohol, melted at 242°.

Analysis (substance dried at 100° in vacuo).

6·412 mgms.; 1·869 mgms. platinum; 30·43 % Pt.
6·927 mgms.; 2·110 mgms. platinum; 30·46 % Pt.
Calculated for betaine platinichloride (mp. 242°); 30·28 % Pt.

The occurrence of the well-known and widely distributed plant base betaine, although not previously observed in rice-polishings, did not surprise us, and we subsequently obtained some interesting results on this point which will be described later.

The alcoholic solution after separation of the betaine hydrochloride was fractionated with platinum chloride but nothing further than small quantities of choline and betaine was isolated.

Method II. (*Sublimate Fraction.*)

The other half of the filtrate from the decomposition of the sublimate precipitate was acidified with sulphuric acid until it contained 5 % of the acid and was then precipitated with phosphotungstic acid. The precipitated phosphotungstates were then treated by a method which we are now using to a considerable extent in our investigations, namely extraction with acetone[1].

It has already been shown that the phosphotungstates vary a great deal in their solubilities in acetone or dilute acetone and advantage has been taken to some extent of this fact by Wechsler [1911]. So far as we know, however, there is no published record of any attempt to employ the different solubilities of the phosphotungstates as a means of separation.

We are, at the present time, making an extensive examination of these solubilities in the hope that by this means a new and useful process may be added to biochemical methods. Preliminary results, a few of which are contained in this communication, lead us to believe that the method may, when fully worked out, be of considerable value, but we cannot pass a verdict until we have completed our investigation of the subject.

In this particular case the phosphotungstates were ground up in a mortar in the cold with acetone until the extract ceased to possess a brown colour, when the insoluble residue was removed by filtration and washed with acetone. We find that extraction in the warm is undesirable, as it generally results in the formation of an extremely sticky mass which is very difficult to deal with, and we have always found that a far better separation is effected when the extraction is carried out at room temperature.

By this means, we obtained a deep brown solution of acetone-soluble phosphotungstates and a small residue insoluble in acetone.

Acetone-soluble phosphotungstates from the sublimate fraction. The deep brown solution was decomposed by shaking well with an excess of a saturated aqueous solution of lead acetate. After standing, the precipitated lead phosphotungstate was filtered off and the filtrate freed from excess of lead by means of sulphuretted hydrogen. The clear solution thus obtained was rendered free from sulphuretted hydrogen and fractionated, as in Method I, by means of silver nitrate, and silver nitrate and baryta into a purine fraction, a histidine fraction, an arginine fraction, and then, after removal of the excess of baryta and silver, into a final phosphotungstic acid fraction.

[1] This method was used for the first time in the fractionation of yeast and cod-liver oil by Funk, shortly to be published.

The purine fraction on decomposition yielded the same purine that we had previously encountered and which we believe was guanine, but here, again, insufficient was obtained to render the identification absolute.

Nothing definite could be isolated from the histidine and arginine fractions, which were very small.

The final phosphotungstic acid precipitate yielded on decomposition practically pure choline, and no betaine could be recognised or isolated from this fraction.

Acetone-insoluble phosphotungstates from the sublimate fraction. This residue, weighing 40 grams, was decomposed by lead acetate, the filtrate freed from excess of lead by sulphuretted hydrogen, acidified with hydrochloric acid and evaporated to a thick syrup under diminished pressure at 30°.

As no crystalline derivative could be isolated directly from this syrup, a sublimate salt was made and recrystallised from water. On decomposition it yielded a hydrochloride, which was soluble in alcohol and melted sharply at 263°.

A platinum double salt was prepared, which, after one recrystallisation from dilute alcohol, melted at 253° and after drying at 100° C. for two hours in vacuo gave the following figures on analysis:

14·896 mgms.; 4·478 mgms. platinum; 30·07 % Pt.
10·826 mgms.; 3·245 mgms. platinum; 29·98 % Pt.
Calculated for nicotinic acid platinichloride (mp. 253°); 29·72 % Pt.

The mother liquor from the mercurichloride precipitation on decomposition yielded a platinichloride which was readily identified as betaine platinichloride.

Apparently, in the investigation of this fraction by utilising the separation of the phosphotungstates by means of acetone, into an acetone-soluble and an acetone-insoluble portion, we have been able to separate the choline practically quantitatively from the betaine and nicotinic acid, thereby rendering the identification of the small quantity of the latter possible by removing the choline into another fraction.

We have therefore found the Sublimate Fraction to contain mainly choline together with small quantities of betaine, nicotinic acid and probably traces of guanine. It is of interest to note that nicotinic acid has been isolated from this fraction, which it has already been shown contains a small amount of the curative substance. The parallel occurrence of nicotinic acid

and the curative substance has already given rise to the suggestion made by one of us [Funk, 1913, 2] that nicotinic acid may be a degradation product of the curative substance. This point will, however, be referred to again later.

2. Investigation of the Purine Fraction.

This fraction, weighing 47 g., was decomposed by means of sulphuretted hydrogen and the clear filtrate from the silver sulphide concentrated under diminished pressure at 30°. After concentration to half bulk, the excess of nitric acid in the solution was quantitatively removed by means of nitron and after removal of the nitron nitrate the concentration continued to dryness. During the concentration two crops of a sandy solid were obtained which were removed by filtration. This substance, which was impure, weighed 0·3 g. and was recrystallised from water, in which it is very insoluble. Thus purified, it showed all the reactions of adenine, but did not give very concordant figures on analysis by the micro-combustion method of Pregl.

The picrate closely corresponded with adenine picrate.

Analysis (dried at 100° C. in vacuo for two hours).

2·740 mgms.; 0·717 cc. N. at 21° C. and 770 mm.—30·62 % N.
Calculated for adenine picrate .. .. .. 30·77 % N.

From the tarry residue left after the separation of the adenine and further concentration of the mother liquor, nothing very definite could be isolated except a very small quantity of a crystalline picrate melting, without further purification, at 280°. The amount, however, was far too small to admit of any examination.

The purine fraction consists mainly of adenine together with possible traces of other purines.

3. Investigation of the Silver Nitrate-Baryta Fraction.

This fraction, which, as originally shown by Funk, contains practically the whole of the curative substance, has already been very exhaustively investigated and the results of the investigation published elsewhere [Funk, 1913, 2]. In that case 2·5 g. of a highly active substance were obtained, which after one recrystallisation from dilute alcohol gave a white substance crystallising in small needles which melted at 233°.

This substance did not give the Millon test nor the uric acid or phenol

tests, it was sparingly soluble in water and on analysis, using the micro-combustion method of Pregl, the following figures were obtained:

	C.	H.	N.
(1)	58·80 %	3·93 %	10·58 %
(2)	58·89 %	3·89 %	10·64 %

From these figures the formula $C_{26}H_{20}O_9N_4$ was arrived at to represent this substance.

Calculated figures for $C_{26}H_{20}O_9N_4$:

C.	H.	N.
58·62 %	3·79 %	10·53 %

The substance did not yield all its nitrogen by Kjeldahl's method.

From the mother liquor of this substance another crop of crystals was obtained which were somewhat less coloured and apparently more soluble in water.

After recrystallisation from dilute alcohol, this substance melted at 234° and no depression of the melting point was observed when melted together with a sample of synthetic nicotinic acid.

Analysis gave the following figures:

	C.	H.	N.
(1)	58·37 %	3·93 %	11·11 %
(2)	58·45 %	4·06 %	10·97 %

Calculation for nicotinic acid:

C.	H.	N.
58·30 %	4·08 %	11·34 %

The identity of the product from rice-polishings was further established by the melting point and analysis of the picrate.

Barger [1914] has remarked that the substance described with melting point 233° to which the provisional formula $C_{26}H_{20}O_9N_4$ had been given, showed a very close resemblance to nicotinic acid both as regards its melting point and its elementary composition, and he suggested that the possibility was not excluded that this body might in reality be merely nicotinic acid contaminated with a small quantity of a highly active substance rich in carbon.

It must be remembered that the substance $C_{26}H_{20}O_9N_4$ was not described as being curative, and, in fact, it was found that it has no greater curative action than nicotinic acid. With regard to the suggestion that the compound $C_{26}H_{20}O_9N_4$ is identical with nicotinic acid, our work goes to show that it is quite correct.

Both substances crystallise in the same form and the crystals melt at 232–234°. A mixed melting point determination gave the figure 232°.

The two picrates were prepared and both presented the same crystalline appearance. They both melted at 215°, and a mixed sample also melted at 215°. Their copper salts were also prepared and were identical in appearance.

Analysis of copper salt of $C_{26}H_{20}O_9N_4$ dried at 100° C. in vacuo:

(1) 19·100 mgms.; 4·180 mgms. CuO; 20·13 % Cu.
(2) 19·060 mgms.; 4·93 mgms. CuO; 20·65 % Cu.
Calculated for the copper salt of nicotinic acid; 20·67 % Cu.

The identity of the substance previously described as $C_{26}H_{20}O_9N_4$ with nicotinic acid could now be doubted no longer, and a careful examination of the mother liquid of these two crops of crystals did not result in the isolation of any other substance than nicotinic acid. All trace of the curative substance had disappeared, and a fraction, which originally had shown very marked curative properties, now consisted of nothing other than nicotinic acid, which possesses very slight action.

All attempts which we have made to isolate the elusive curative substance from this fraction, in which it originally occurs, have failed, and we can only hope that one or other of the new methods which we are testing at the present time, will give a process by which it can be isolated before it has time to become inactive.

4. Investigation of the Phosphotungstic Acid Fraction.

This fraction, weighing 195 g., was treated by grinding in a mortar with acetone, as already described, until the acetone extract ceases to be coloured.

The two fractions thus obtained were then investigated separately.

Acetone-insoluble sub-fraction of fraction 4. This weighed 25 g. and was decomposed by means of lead acetate. The filtrate, after being freed from lead, was acidified with hydrochloric acid and concentrated at low temperature and pressure to a syrup. As no definite crystalline substance could be obtained from this syrup, alcoholic mercuric chloride was added, and the precipitated mercury chloride salt removed by filtration.

On decomposition the mercurichloride yielded a small amount of a rather hygroscopic hydrochloride from which a well-defined platinichloride was prepared.

After one recrystallisation from dilute alcohol the platinum salt melted at 232°.

Analysis (dried at 100° C. in vacuo).

(1) 11·670 mgms.; 4·024 mgms. platinum; 34·48 % Pt.
(2) 7·155 mgms.; 2·463 mgms. platinum; 34·42 % Pt.

After another recrystallisation from dilute alcohol it melted at 234°.

(3) 4·235 mgms.; 1·453 mgms. platinum; 34·39 % Pt.

The small quantity of this salt which we obtained prevented us from making any further examination of it, and we have so far been unable to identify it.

The filtrate from the platinum double salt, together with the mother liquors from its recrystallisation, were decomposed and attempts made to prepare a gold salt from the residue, but without success.

The filtrate from the mercuric chloride precipitation was decomposed and concentrated at low temperature and pressure to dryness. A very small residue of impure crystalline hydrochlorides was obtained which, however, contained inorganic substances. Alcohol extraction removed the organic portion, which yielded a small quantity of a platinichloride melting with decomposition at 280°, but the quantity obtained was far too small to admit of purification or examination.

Acetone-soluble sub-fraction of fraction 4. This weighed 175 g. and was decomposed in the usual manner by means of lead acetate. The filtrate, after freeing from lead, was concentrated at low temperature and pressure to a thick syrup. Nothing crystallised from this residue on standing, so saturated aqueous picric acid was added. A small amount of a brownish yellow semi-crystalline picrate separated out and was removed by filtration. This picrate was very insoluble in cold water and very little more soluble in hot. It was recrystallised from water and separated as a golden brown powder. It darkened on heating at 200° but showed no melting point below 289°. Determinations of its content of picric acid were made by the nitron method on a sample dried at 100° in vacuo for two hours. The figures obtained were:

(1) 0·0749 g.; 0·1388 g. nitron picrate; 78·45 % picric acid.
(2) 0·0395 g.; 0·0732 g. nitron picrate; 78·43 % picric acid.

Portions of the picrate and of the mother liquid from its recrystallisation were decomposed with ether and hydrochloric acid, and yielded upon

concentration to dryness a very small residue of the base in the form of its hydrochloride.

It was itself insufficiently pure to examine, and attempts to prepare a platinichloride, a gold salt or a picrolonate were all unsuccessful.

Guanidine picrate melts at 315°, exhibits similar solubilities to the compound we isolated from rice, and contains 79·10 % of picric acid. It is possible that we were here dealing with guanidine picrate, but we were unable to be sure of this point because of the very small quantity which was at our disposal.

The filtrate from the precipitation of this picrate was rendered free from picric acid by means of acidification with hydrochloric acid and shaking out with ether, and was then concentrated at 37° under diminished pressure. During the concentration a crystalline substance separated out and was filtered off, after which the concentration was continued to dryness.

The crystalline substance, which had separated out during the concentration, was recrystallised from dilute alcohol and separated out from that solvent in the form of small needles. Without further purification it melted sharply at 235°.

The isolation of this substance was of considerable interest to us, as we have encountered it several times in the investigation of rice-polishings. If the total phosphotungstate precipitate from rice-polishings is decomposed by means of lead acetate, and the filtrate, after freeing from lead and acidifying with hydrochloric acid, concentrated to dryness, a semi-crystalline residue of hydrochlorides is obtained. If now, this residue be extracted and recrystallised from dilute alcohol, the same crystalline substance melting at 235° is obtained.

On several occasions we have isolated this substance, and, after having collected some few grams, we decided to investigate it. It was recrystallised once again from alcohol and then melted sharply at 234°.

It was noticed that on drying, the crystals often—although not in every case—developed a fine blue colour. Some crystals apparently developed the colour to a far greater extent than others, and we found that the production of the colour was due to a photochemical action. The colour disappears when the crystals are dissolved, and when they separate out again they are perfectly white, so long as they are covered by the solvent. When they are removed from the solution and placed in the light they quickly develop the colouration again, and we have found them to develop this colouration after as many as six recrystallisations.

We have not yet satisfied ourselves as to the cause of the colouration, but believe it is in some way the result of the action of light upon minute traces of tungsten compounds which have been persistently retained from the phosphotungstic acid decomposition. This however was never observed in other fractionations.

The substance which we had now obtained in an apparently pure condition was analysed by micro-combustion. Concordant figures could not, however, be obtained on duplicate analyses, despite further purification of the substance by recrystallisation from dilute alcohol. The substance did not give all its nitrogen when estimated by Kjeldahl, and in many ways resembled nicotinic acid hydrochloride, which, however, as prepared from nicotinic acid, always melts at 263°.

A well-defined platinum double salt was prepared, which after one recrystallisation from dilute alcohol melted sharply at 253°, the theoretical melting point for the platinichloride of nicotinic acid, and no depression of the melting point was observed when a mixed sample of the two platinichlorides was melted.

Analysis (dried at 100° C. for two hours in vacuo).

(1) 7·746 mgms.; 2·318 mgms. platinum; 29·86 %.
(2) 10·479 mgms.; 3·136 mgms. platinum; 29·93 %.
Calculated for platinichloride of nicotinic acid; 29·72 %.

The free base was liberated from the hydrochloride by means of silver acetate, and from the product thus obtained pure nicotinic acid was isolated, which was in every respect identical with the synthetic acid.

We were unable to explain the difference in the melting points of what we imagined were two distinct modifications of the hydrochloride of nicotinic acid, and could not account for the inconstant figures obtained on analysis of the hydrochloride melting at 235°, until an attempt to recrystallise the latter compound from absolute alcohol provided us with a clue, which, after investigation, resulted in the clearing up of the whole matter.

It was found that the compound melting at 235° was not all equally soluble in absolute alcohol and that a portion of it was very insoluble in cold alcohol.

This less soluble portion was fractionated with cold alcohol, and eventually a white crystalline substance was obtained which was very insoluble in cold alcohol and which after one recrystallisation from dilute alcohol melted sharply at 245°. A platinichloride was prepared and formed a fine,

orange-coloured, needle-shaped, crystalline compound which after one recrystallisation from dilute alcohol melted at 242°.

Analysis. 13·133 mgms.; 3·998 mgms. platinum; 30·44 % Pt.
Calculated for betaine platinichloride (mp. 242°); 30·28 % Pt.

It also gave a picrate which melted at 182° and was identical in every respect with betaine picrate.

We then realised that in the compound melting at 235° we were dealing with some complex, probably of the nature of mixed crystals, of nicotinic acid hydrochloride and betaine hydrochloride.

We therefore decided to effect a separation of the two constituents, and after several attempts found that this could be quantitatively carried out by utilising the insolubility of the copper salt of nicotinic acid.

The hydrochloride was dissolved in water and was warmed on the water bath at 60° for several hours with excess of a saturated solution of copper acetate until no further deposition of any copper salt occurred. The precipitated copper salt and the clear blue mother liquor were then separated, and decomposed individually by means of sulphuretted hydrogen.

The pale green, amorphous copper salt on decomposition and concentration of the filtrate from the copper sulphide yielded a crystalline hydrochloride, which after one crystallisation from dilute alcohol was shown to be identical with nicotinic acid hydrochloride. It melted at 264°, its picrate at 214°, and none of these derivatives showed any depression in its melting point when melted with the corresponding derivative of nicotinic acid.

The bright blue-green filtrate from the insoluble copper salt of nicotinic acid was decomposed in a similar manner, and yielded a hydrochloride, very insoluble in cold alcohol, which after a single recrystallisation from alcohol melted at 245° and was identical with betaine hydrochloride. Its picrate melted at 181°, and no depression of this melting point was observed on melting it together with a sample of betaine picrate. Its gold salt melted at 209° but on recrystallisation from 1 % hydrochloric acid, the other form melting at 247° crystallised out.

Analysis. 11·884 mgms. gold salt; 5·119 mgms. gold; 43·09 % Au.
Calculated for betaine aurichloride (mp. 247°); 43·11 % Au.

We also tried other methods of separating the two constituents of this complex, but found no method so useful as that which we have described in which we used copper acetate.

Alcohol fractionation removed the betaine hydrochloride gradually, but

we were not able to prepare a pure specimen of nicotinic acid by this process. Fractionation of the picrate of the complex also gave us fairly pure betaine picrate in the less soluble fractions, but we were unable to obtain nicotinic acid picrate free from betaine picrate.

It was of interest to us to try the curative action of the complex hydrochloride, and accordingly doses were administered by intra-muscular injection into pigeons suffering from experimental beri-beri, but we did not observe any arrest of the symptoms or of the onset of death. Betaine salts were likewise examined and found to be entirely without curative action.

The residue left after concentration of the mother liquors, from which the complex hydrochloride melting at 235° had separated out, was then examined, but only yielded a mercurichloride. This was decomposed and found to consist of choline as identified by its platinichloride, melting at 242°.

Analysis (dried at 100° C. in vacuo).

7·643 mgms.; 2·439 mgms. platinum; 31·91 % Pt.
Required for choline platinichloride; 31·73 % Pt.

The filtrate from this small quantity of choline mercurichloride on decomposition yielded a small residue which gave a platinichloride. After a single recrystallisation it melted at 237°.

Analysis (dried at 100° C. for two hours in vacuo).

(1) 8·418 mgms.; 2·906 mgms. platinum; 34·57 % Pt.
(2) 15·092 mgms.; 5·199 mgms. platinum; 34·45 % Pt.

This platinichloride seemed, despite its somewhat higher melting point, identical with that which had been isolated in the acetone-insoluble subfraction of this fraction. Here again we obtained insufficient to render much further examination possible. A small quantity was decomposed and the free hydrochloride prepared. No gold salt, picrate or picrolonate could be obtained, and consequently we have been unable to prepare any other derivative by means of which it might be identified.

There is the bare possibility that it might be the platinichloride of choline contaminated with traces of potassium platinichloride, which it is known are persistently retained by the choline salt, but it did not give the periodide test for choline.

No other substance could be isolated from this fraction, from which we have separated betaine, choline, nicotinic acid, a picrate that resembles guanidine picrate, and the platinichloride of a base which we have been unable to identify.

We may now summarise our findings, and it is here that a convenient point is reached at which to discuss some of the results of previous workers.

From an exhaustive fractionation of the phosphotungstic acid precipitate from the alcoholic extract of fat-free rice-polishings, we have isolated comparatively large amounts of choline, nicotinic acid and betaine, together with small amounts of the purines, guanine and adenine and traces of a substance which may be possibly guanidine.

Choline and nicotinic acid have been found by the majority of the previous workers, because they occur in larger amounts than any of the other constituents. We have, whilst carrying out this work, paid particular attention to the published work of Suzuki, Shimamura and Odake [1912] in the hopes that we might be able to find some corroboration of their results. We have, however, with a few exceptions, been entirely unable to do so. By decomposition of the phosphotungstic acid precipitate from rice-polishings with baryta and subsequent concentration of the filtrate, they obtained a product which was highly curative in action and which they designated "Rohoryzanin I."

In spite of the fact that it is quite obvious that "Rohoryzanin I" is a complex mixture of basic substances, they boiled it with 3 % hydrochloric acid for two hours and then looked on the products they isolated from the fluid as hydrolytic products of Rohoryzanin.

From this hydrolysis they obtained two acids, which they termed α and β acids, choline, nicotinic acid, and glucose. Thus they said that from 100 parts of Rohoryzanin there were obtained on hydrolysis 10 parts of the α and β acids, 30 parts of choline and nicotinic acid and 23 parts of glucose.

The two acids were obtained by allowing the hydrolysed solution of Rohoryzanin to cool, when golden brown coloured crystals separated out. These were separated, washed with a small quantity of cold water, and recrystallised from alcohol, when two different products, separated by their different solubilities in alcohol, were obtained, and from the elementary analyses the formulae $C_{10}H_8O_4N$ and $C_{18}H_{16}O_9N_2$ were given to the two acids.

Funk (1913, 1) tried to prepare these two bodies, following exactly the method described by Suzuki and his co-workers, but has never yet succeeded in obtaining them. Whenever the product which these authors termed "Rohoryzanin" was hydrolysed a clear solution was obtained which did not deposit any crystalline substance whatever on cooling. From this solution on concentration we have now separated the hydrochlorides of nicotinic acid and betaine by their different solubility in alcohol, but we can hardly believe

that these were the substances described asthe acids $C_{10}H_8O_4N$ and $C_{18}H_{16}O_9N_2$. Unfortunately, beyond giving a few solubilities and reactions the authors do not give much information about these two products.

The Japanese workers, from their descriptions of "Rohoryzanin," were apparently under the delusion that they were dealing with a single substance and give several reactions for it. They even claim that a characteristic reaction for "Rohoryzanin" is the *p.*-diazobenzenesulphonic acid test.

It was found that the substance described as "Rohoryzanin" does give the "diazo" reaction, but we believe that the purine content of this crude mixture of bases is quite sufficient to account for a positive reaction being obtained.

Suzuki, Shimamura and Odake also describe nicotinic acid, choline and glucose amongst what they term "die Spaltungsprodukte des Rohoryzanins," obtained by hydrolysis with 3 % hydrochloric acid.

With regard to nicotinic acid, we ourselves lean towards the belief that its occurrence in rice-polishings has some relationship to the occurrence of the curative substance, and that it may possibly even be a degradation product of the active body, but in the case of choline we firmly believe that it exists as such, and in comparatively large amounts, in the polishings.

The workers, whose work we are criticising so fully, purified their "Rohoryzanin I" by means of precipitation with tannin, and thereby obtained another product which they called "Rohoryzanin II," and which was three times more active than the original substance. The yield, however, was very small.

From a concentrated aqueous solution of "Rohoryzanin II," they obtained on addition of picric acid a precipitate of a golden-brown crystalline picrate. They separated it and found that it contained as an impurity nicotinic acid picrate, due to "Rohoryzanin II" containing impurities of nicotinic acid.

They succeeded in preparing this picrate, "Oryzanin picrate," free from nicotinic acid picrate and further purified it by recrystallisation from acetone.

Thus prepared, it was described as a golden-brown picrate crystallising in microscopic needles. It is insoluble in ether and petroleum ether, rather insoluble in water, more soluble in hot water, alcohol or ether. The yield was very poor, and very little detail is given beyond the few solubilities given above. It was highly active when administered to pigeons suffering from experimental beri-beri and was regarded by the discoverers as the curative substance of rice-polishings in the form of its picrate.

We have attempted to prepare this substance but have not yet succeeded

in doing so. Among the substances which we isolated from rice-polishings was one picrate which corresponded in many respects with the picrate described by the Japanese authors. This picrate, already described in a previous portion of the paper, we believe to have been guanidine picrate. Whether this is so or not we are not sure, but we certainly know that it possesses no curative action whatever.

Not one of the picrates which we isolated demonstrated any such action, and we have been quite unable to confirm the isolation of the curative substance in the form of its picrate as described by Suzuki and his collaborators.

Summary.

1. We have carried out an exhaustive fractionation of the phosphotungstic acid precipitate from an alcoholic extract of rice-polishings.

2. We have once again confirmed the presence of considerable amounts of choline and nicotinic acid in the polishings, and have also detected the presence of betaine, adenine, guanine and possibly guanidine. Small amounts of other substances were isolated but in quantities too small to admit of identification.

3. We have repeated the work of the Japanese authors Suzuki, Shimamura and Odake, who claimed to have isolated the curative substance in the form of its picrate, but have been unable to confirm this finding, as well as some other portions of their work.

4. We have ourselves failed to isolate the curative substance which occurs in rice-polishings. It is apparently decomposed during the fractionation and all trace of it is lost.

REFERENCES.

Barger, G. (1914). *The Simpler Natural Bases*, p. 112 (Longmans Green and Co.).
Funk, C. (1911). *J. Physiol.*, **43**, 395.
— (1913, 1). *J. Physiol.*, **45**, 489.
— (1913, 2). *J. Physiol.*, **46**, 173.
Suzuki, Shimamura and Odake (1912). *Biochem. Zeitsch.*, **43**, 89.
Wechsler, E. (1911). *Zeitsch. physiol. Chem.*, **73**, 138.

LXI. A COMPARISON BETWEEN THE MOLECULAR WEIGHTS OF PROTAGON AND OF THE PHOSPHATIDE AND CEREBROSIDES OBTAINABLE FROM IT.

By ALEXANDER LYALL PEARSON.

From the Chemical Laboratory of the Physiology Department, Edinburgh University.

(*Received Nov. 6th, 1914.*)

Introduction.

The following observations were carried out with the object of obtaining fresh and conclusive information concerning the question whether protagon is a mixture of phosphatides and cerebrosides or whether it contains these substances in chemical combination. All authors are agreed that it is possible to prepare from protagon two cerebrosides, namely cerebrin and homocerebrin (phrenosin and kerasin of Thudichum), and a phosphatide, sphingomyelin. But while according to Thudichum and his followers protagon is simply a mixture of these three substances, together perhaps with a number of other simpler substances, the followers of Gamgee maintain that protagon contains these substances in chemical combination which is easily broken up with the liberation of the constituent phosphatide and cerebrosides, just as haemoglobin is easily broken up into globin and haematin.

Hitherto all attempts to decide this question have been made by studying the behaviour of protagon on the one hand and of cerebrin, homocerebrin and sphingomyelin and mixtures of the three on the other hand towards certain solvents such as alcohol, chloroform, pyridine and others. But this line of argument has not only failed to bring about an agreement but has actually led to an impasse.

The observations of one group of authors are incompatible with those of another. Thus Thudichum and Rosenheim and Tebb state that protagon has

an indefinite and variable melting point, contains relatively large amounts of potassium, and completely alters its composition on recrystallisation from large volumes of alcohol; in other words, its behaviour is that of a mixture of a phosphatide and cerebrosides. On the other hand, on subjecting these statements to re-examination Gamgee, Roscoe, Baumstark, Ruppel, and Cramer find that protagon has a definite melting point, is free from potassium and retains its composition on recrystallisation from large and small volumes of alcohol; in other words its behaviour, according to these authors, is not that of a mixture of a phosphatide and cerebrosides. There is therefore hardly any common ground on which a discussion as to the nature of the substance in question is possible along these lines. Indeed the only conclusion an impartial observer can draw, is that the substance investigated by Thudichum and his followers is not identical with the one studied by Gamgee and his followers. Nor are these contradictions to be found only between the statements of those who attack and those who defend the existence of protagon as a definite compound. The observations of Posner and Gies that protagon retains its composition when dissolved in warm alcohol and cooled immediately, however large the volume of alcohol, is diametrically opposed to the statements of Rosenheim and Tebb. No explanation has ever been offered by the latter observers to account for this contradiction, and it is difficult to understand how these authors can quote each other in support of their views.

A new and more decisive line of argument is necessary in order to settle this question. It is offered in this paper and is as follows. The chemical composition of cerebrin and homocerebrin is fairly well known owing mainly to the work of Thudichum, Kossel and Freytag, Thierfelder and Levene and Jacobs. Thierfelder [Kitagawa and Thierfelder, 1906] from his observations on the products of acid hydrolysis of cerebron, which is either identical with or closely allied to cerebrin, has calculated the formula $C_{48}H_{93}O_9N$ which has also been accepted by Levene and Jacobs [1912] as the result of their analysis. The molecular weight of cerebron according to this formula would therefore be 827. In the case of the closely allied homocerebrin the molecular weight has been determined directly by means of the elevation of the boiling point by Kossel and Freytag and was found to lie between 945 and 1027. In the case of sphingomyelin our knowledge depends mainly on the statement of Thudichum, who from a study of the products of hydrolysis gives to it the formula $C_{52}H_{104}O_9N_2P$. The molecular weight of sphingomyelin would be, accordingly, 931.

The three substances which can be obtained from protagon therefore have molecular weights of such an order of magnitude that, if they follow Raoult's law, a definite elevation of the boiling point should be noticeable in solutions of moderate concentrations (3 %–5 %) of these substances, especially if one uses a solvent with a relatively high constant such as chloroform. One gram of a substance with the molecular weight 1000 would, in a 4 % solution in chloroform, elevate the boiling point by about 0·10°. If therefore protagon were a mixture of these substances it should, with the concentrations given, produce a distinct elevation of the boiling point of chloroform.

On the other hand, if protagon contained these substances in chemical combination it would have a molecular weight at least approximating to 3000. It may, of course, be considerably higher; in fact Cramer calculated from the amount of sulphur in protagon a molecular weight of 5778. If therefore protagon were a combination and not a mixture of cerebrosides and phosphatides the boiling point of a 3 % to 5 % chloroform solution of protagon should show either no elevation at all of the boiling point of chloroform or only a very slight one compared with that produced by cerebrin, homocerebrin, sphingomyelin or mixtures of these substances.

Observations on the boiling point of chloroform solutions of these substances by means of Beckmann's method will therefore give a final and decisive answer to the question whether protagon is a chemical combination or a mixture of phosphatides and cerebrosides. And since with substances of high molecular weights the determination of the molecular weights can only be approximate, it may be pointed out that this answer is not dependent on the exact numerical evaluation of the molecular weights nor upon slight quantitative differences between them, but upon differences of such an order of magnitude as to be qualitative.

Experimental.

The observations were made by Beckmann's method with an apparatus having an electrical heating device. The amount of chloroform used was measured in every case by volume, not by weight, and amounted to 25 cc. The chloroform was "chloroform from chloral" except in one case when "chloroform from acetone" was used. In almost every case a reading of the boiling point of the pure solvent was taken both before and after the experiment in order to be able to correct for changes due to variations in

barometric pressure. The boiling point of a solution was taken as the point where three successive readings at intervals of five minutes gave constant results.

Protagon was prepared from ox brain by the method of Wilson and Cramer: after completely exhausting the fresh minced brain tissue by repeated extraction with cold acetone and cold ether at room temperature the residue was extracted rapidly with boiling alcohol. Two alcohol extracts only were made. The white precipitate, obtained on cooling the alcoholic extract, was twice recrystallised. No more than ten ox brains were worked up at any one time. The process was repeated with new quantities of fresh ox brains until about 12 g. of dry protagon had been obtained in the form of a snow-white pulverulent powder. It is not advisable to work with large quantities of brain tissue.

Part of the protagon obtained in this way was used for the preparation of cerebrin and homocerebrin, by boiling it with baryta water. This method, which was used originally by Parcus and by Kossel and Freytag, has been re-introduced again recently by Lorrain Smith and Mair [1910] and by Loening and Thierfelder [1911]. Protagon is made into a fine emulsion with saturated baryta water, heated under a reflux condenser in a vigorously boiling water bath for one hour and the mixture filtered. The residue, after boiling with a mixture of alcohol and acetone, is repeatedly extracted with boiling acetone from which on cooling a mixture of cerebrin and homocerebrin separates out. The mixture thus obtained was recrystallised twice from boiling acetone and was obtained eventually in the form of a white powder more granular in appearance than protagon.

Since it was the object of these observations to compare the behaviour of protagon with that of a mixture of the cerebrosides which can be prepared from it, the separation of the two cerebrosides was not carried out. The observations made with protagon, and with the mixture of cerebrin and homocerebrin respectively, are recorded in Tables I and II.

These observations show clearly that the size of the protagon molecule is very much larger than that of the molecules of cerebrin and homocerebrin. Indeed the protagon molecule is so large that even in concentration exceeding 5% it fails to produce an elevation of the boiling point of chloroform. Now it is possible to calculate from the amount of galactose liberated from protagon, cerebrin, and homocerebrin respectively that *one gram* of protagon contains 0·6 to 0·7 gram of cerebrosides and 0·4 to 0·5 gram of sphingomyelin. Even if one assumed that sphingomyelin had a very much larger molecule

than the data of Thudichum would indicate, or if one assumed that for some reason or other it did not follow Raoult's law, perhaps by aggregating in solution into a colloidal form,—even then the elevation of the boiling point produced by *one gram* of protagon ought to be at least as great as that produced by 0·6 to 0·7 gram of the cerebrosides obtained from it. As a matter of fact, however, 0·7 gram of a mixture of cerebrin and homocerebrin

TABLE I.

Boiling point of chloroform solutions of protagon.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	e	Barometer
		—	59·62		
I.	25	0·6664	59·62	0	734
		—	59·62		
		—	59·92		
II.	25	0·9730	59·92	0	742
		—	59·92		
		—	60·50		
III.[1]	25	0·9430	60·45	−0·05	not read
		1·5920	60·45	−0·05	
		—	60·50		

[1] In observation No. III chloroform from acetone was used.

TABLE II.

Boiling point of a chloroform solution of a mixture of cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	e	Barometer
		—	59·50		
		0·7305	59·53	+0·03	
IV.	25	1·5140	59·63	+0·13	728
		—	59·50		

is sufficient to produce a distinct elevation of the boiling point of 25 cc. of chloroform, while even double the quantity of protagon is incapable of producing that effect. It follows, then, that the observations recorded in Tables I and II are incompatible with the view that protagon is a mixture of sphingomyelin, cerebrin and homocerebrin.

It will be noted that the elevation of the boiling point produced by cerebrin and homocerebrin increases with increasing concentration out of proportion to the amounts of substance added. This irregularity has no

bearing on the problem under discussion but it is of some general interest since a similar phenomenon has been observed in the case of other substances with large molecules, for instance in the case of colloidal solutions of silicic acid, tungstic acid and molybdic acid. It will also be noted in one case (Experiment No. III) that the boiling point of a chloroform solution of protagon instead of remaining constant showed a slight diminution, which, however, was not increased on adding more protagon. Since in this case a less pure sample of chloroform ("chloroform from acetone") was used, the irregularity may probably be referred to this fact.

Although a chloroform solution of protagon shows no elevation of the boiling point, yet the presence of protagon does not hinder the boiling point of chloroform being affected by the addition of another substance. This is shown by the fact that the addition of a weighed quantity of naphthalene to 25 cc. of a chloroform solution containing 0·6664 gram of protagon produced an increase of the boiling point commensurate with its molecular weight, 128. Thus the addition of 0·4240 g. naphthalene gave an elevation of the boiling point $e = 0{\cdot}37°$, molecular weight calculated, 119. The further addition of 0·4306 g. naphthalene produced a further elevation, $e = 0{\cdot}37°$, molecular weight calculated, 121.

In order to compare the molecular size of sphingomyelin with that of cerebrin, homocerebrin, and protagon, sphingomyelin was prepared by heating crude protagon with pyridine to 50° for 20 minutes. The precipitate which falls out on cooling the pyridine is removed by filtration. After washing with cold alcohol and drying in vacuo it had a phosphorus percentage of 2·3 %; it represents according to Rosenheim and Tebb [1910] sphingomyelin with a slight admixture of cerebrosides. The pyridine filtrate from sphingomyelin when poured into an excess of acetone gives a precipitate consisting of cerebrosides with a slight admixture of sphingomyelin. The sphingomyelin obtained in this way was not freed from its slight admixture of cerebrosides, since the object of these observations is to obtain comparative values for protagon on the one hand, and the substances or mixtures of the substances which can be obtained from it, on the other.

The observations made with sphingomyelin prepared in this way are given in Table III. It will be seen that sphingomyelin produces a distinct elevation of the boiling point of chloroform, thus indicating that the size of the molecule of sphingomyelin is of about the same order of magnitude as that of cerebrin and homocerebrin and very much smaller than that of protagon. The observations with sphingomyelin, therefore, confirm the

conclusions arrived at from the observations with cerebrin and homocerebrin.

The preparation of sphingomyelin and the cerebrosides from protagon by heating the latter with pyridine made it possible to compare protagon with a mixture of sphingomyelin and cerebrosides in approximately the

TABLE III.

Boiling point of chloroform solution of sphingomyelin (with slight admixture of cerebrosides).

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	ϵ	Barometer
		—	60·56		
V.	25	0·6842	60·60	+0·04	754
		—	60·35		
VI.	25	1·0948	60·42	+0·07	752

TABLE IV.

Boiling point of a chloroform solution of a mixture of sphingomyelin, cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	ϵ	Barometer
		—	60·35		752
		1·0948 (sphingomyelin)	60·42	+0·07	
VII.	25	+ 0·7210 (cerebrosides)	60·45	+ +0·03	
		+ 0·7196 (cerebrosides)	60·47	+ +0·02	
		—	60·25		749

Note that in this table the changes in the elevation of the boiling point produced by the successive addition of sphingomyelin and cerebrosides are given separately with reference to each single addition and not collectively. For a detailed discussion see text.

same proportions in which they can be obtained from it. For that purpose the chloroform solution of sphingomyelin obtained in Experiment No. VI, Table III, was used. After having obtained a constant value for the boiling point of this solution the cerebrosides obtained by the pyridine treatment described above were added. The results are given in Table IV. There was a rapid fall of barometric pressure on the day on which this observation was carried out. Experiment No. V, Table III, was made on the forenoon of the

same day, Experiment No. VI, Table III, and the observations recorded in Table IV, on the afternoon of that day. It will be seen that the barometer fell from 754 mm. at 9 a.m. to 749 mm. at 3 p.m. There is, of course, a corresponding fall of the boiling point of the pure solvent. Since in the observations recorded in Table IV the boiling points of three different solutions were determined, one hour and a half elapsed between the beginning and the end of these observations, and even during that time a distinct fall in boiling point of the pure solvent was noticed.

Although the barometric variations would tend to diminish any elevation in the boiling point produced by the addition of cerebrosides, there is nevertheless clear evidence of a rise in the boiling point of the chloroform solutions of sphingomyelin on every addition of cerebrosides. Taking the last observations it will be seen that a mixture of about 1·1 gram sphingomyelin and 1·4 gram cerebrosides having a phosphorus percentage of 1 % corresponding to that of protagon produced a total rise of 0·22°. Of this rise 0·07 is due to the sphingomyelin present, and consequently a rise of 0·15 is due to the cerebrosides. This is approximately the same value as that obtained before in the observation recorded in Table II. In other words, the effect produced by a mixture of sphingomyelin, cerebrin and homocerebrin is simply the additive effect produced by the substances constituting the mixture. If protagon were a loose molecular compound of phosphatides and cerebrosides which could be reconstituted by simply mixing these substances together as has been suggested by some writers, the addition of cerebrosides to the solution of sphingomyelin should diminish the elevation of the boiling point produced by sphingomyelin. As a matter of fact the opposite takes place. The observations show then again that protagon is not a mixture of sphingomyelin, cerebrin and homocerebrin, but that it must contain these substances in chemical combination. They also show that it is not possible to reconstitute protagon by mixing together in certain proportions the cerebrosides and phosphatides which can be prepared from it.

Discussion.

The observations recorded in this paper show that the molecule of protagon is very much larger than that of cerebrin, homocerebrin or sphingomyelin. It follows therefore that protagon is not a mixture of these substances but holds them in chemical combination. It is also shown that the behaviour of a mixture of cerebrin, homocerebrin and sphingomyelin is

different from that of protagon and that it is not possible to reconstitute protagon by simply mixing together these simpler substances in certain proportions. This again shows that protagon is not a mixture of phosphatide and cerebrosides. It also shows that protagon is not a loose molecular compound of these substances which is formed when these substances occur together in solution.

While the observations recorded in this paper establish the fact that protagon is a chemical combination and not a mixture of cerebrosides and phosphatides, they do not answer the entirely different and much less important question whether protagon is only one such combination of phosphatides and cerebrosides or a mixture of several such combinations.

Although the essential difference between these two questions is obvious, it is necessary to emphasise it, because they have been confused in the past. Those who held with Gamgee that protagon is not a mixture of phosphatides and cerebrosides have been represented by their opponents as maintaining that protagon is not a mixture; they have been criticised for not bringing forward any evidence to prove that protagon is a single substance, and from their inability to do so it has been argued, not that protagon is a mixture, which might be logical, but that protagon is a mixture of phosphatides and cerebrosides, which is quite illogical. In this way the entire protagon question has become obscured and distorted. It is obvious that the fact that protagon is a chemical combination of cerebrosides and phosphatides does not exclude the possibility that protagon consists of a mixture of several such combinations. This possibility has in fact been pointed out by Kossel and Freytag and by Cramer. The question whether any complex substance isolated from tissues is a single substance or a mixture of homologous substances is as difficult to decide in the case of protagon as in the case of haemoglobin or any other protein. Even in the case of many phosphatides and cerebrosides it is not yet settled. Fortunately no considerations of great theoretical or practical importance depend on the question whether protagon is a single substance or a mixture of several protagons. But considerations of quite a different order of importance are involved in the question whether protagon is a mixture or a chemical combination of cerebrosides and phosphatides. This, however, does not appear to be clearly understood if one may judge from the statements frequently made in the literature, so that a brief indication of the significance of this question is called for.

If the views of Thudichum were correct, phosphatides and cerebrosides

must be considered to be the most complex forms of lipoids present in tissues. Many text-books actually represent the chemistry of lipoids in this way. Now phosphatides are lipoids containing phosphorus but no galactose; cerebrosides are substances containing galactose but no phosphorus. Any substance isolated from tissues which contains both phosphorus and galactose is, from this point of view, considered to be a mixture of phosphatides and cerebrosides and is, therefore, subjected to methods of purification, no matter how drastic, until substances either free from phosphorus or free from galactose are obtained. The substances obtained after such treatment are then considered to exist preformed in the tissues.

We find accordingly substances obtained by boiling brain with baryta water unhesitatingly accepted as preformed constituents of nervous tissue. But since it has been shown in this paper that it is possible to isolate from nervous tissue a substance having a larger molecule than either phosphatides or cerebrosides, and since this substance contains both galactose and phosphorus in its molecule, it is necessary to recognise the existence of a group of lipoids, more complex than either phosphatides or cerebrosides. Since in the case of protagon, which is the best known representative of this group, cerebrosides preponderate in the building up of the molecule, and since protagon in its physical properties resembles cerebrosides and is unlike the typical phosphatides, the term "phosphorised cerebrosides" or briefly "phospho-cerebrosides" as proposed by Cramer [1911] seems most convenient.

The observations recorded in this paper also furnish an answer to the much debated question whether protagon can be decomposed by the application of solvents such as alcohol and pyridine. As pointed out in the introduction, up to the present time the protagon controversy has centred entirely round this question. It has been shown that by the long repeated application of warm alcohol it is possible to prepare from protagon cerebrosides and phosphatides. The fact itself is not disputed; what is in dispute is the interpretation which is to be placed upon it. Thudichum and his followers deny that this process takes place under conditions involving the decomposition of protagon and they describe this process as a "fractional crystallisation." But their statements in regard to this point are always made dogmatically and no evidence is offered in support of them. Gamgee and his followers claim that protagon is easily decomposed by alcohol and Wilson and Cramer have shown that the conditions which make a separation of protagon into phosphatides and cerebrosides possible also produce a distinct change in the physical constants of protagon. More recently Rosenheim and Tebb have

used pyridine as a means of preparing the cerebrosides and sphingomyelin from protagon. Rosenheim [1913] describes the method as a separation by cold pyridine although the process involves a preliminary heating to 40° and claims again that pyridine is an inert solvent and that the process excludes the possibility of decomposition. Again no evidence is offered in support of this claim and it is clear that, if the possibility of a decomposition of protagon by alcohol is admitted, as it is now admitted by Rosenheim, a solvent like pyridine which has a strongly alkaline reaction is even more likely to bring about such a change.

The method of separation by pyridine has been used and described in this paper. The molecular weights of the substances obtained by this method have been examined both when isolated and when mixed together again in the proportions in which they were obtained from protagon. Since protagon has a very much larger molecule than the substances or mixtures of substances which can be obtained from it by the use of pyridine or alcohol, it is evident that the methods used for the preparation of cerebrosides and sphingomyelin from protagon involve a decomposition of protagon.

Summary.

Observations have been made by Beckmann's method on the elevation of the boiling point of chloroform produced by the addition of protagon on the one hand, and of the phosphatide (sphingomyelin) and the cerebrosides (cerebrin and homocerebrin) obtainable from protagon, on the other hand. The observations show that protagon has a very much larger molecule than the phosphatide and cerebrosides mentioned, either when separated or when mixed together. It follows that protagon is neither a mixture of these substances, nor a loose molecular compound formed when sphingomyelin, cerebrin and homocerebrin occur together in solution. The observations prove that protagon is a chemical combination of cerebrosides and a phosphorus-containing lipoid (sphingomyelin). It represents a group of lipoids more complex than either phosphatides or cerebrosides which is most suitably classified by the term "phospho-cerebrosides."

While these observations prove that protagon is a "phospho-cerebroside," *i.e.* a chemical combination of a phosphatide and cerebrosides and not a mixture of these substances, they afford no evidence with regard to the entirely different problem whether protagon is a simple "phospho-cerebroside" or a mixture of several "phospho-cerebrosides."

The expenses of this research have been defrayed by a grant from the Moray Fund of the University of Edinburgh.

REFERENCES.

Cramer (1911). *Biochem. Handlexicon*, **3**, 250.
Kitagawa and Thierfelder (1906). *Zeitsch. physiol. Chem.*, **44**, 366.
Levene and Jacobs (1912). *J. Biol. Chem.*, **12**, 389.
Loening and Thierfelder (1911). *Zeitsch. physiol. Chem.*, **74**, 282.
Lorrain Smith and Mair (1910). *J. Path. Bact.*, **15**, 122.
Thierfelder (1913). *Zeitsch. physiol. Chem.*, **85**, 35.
Rosenheim and Tebb (1910). *J. Physiol.*, **41**, Proceedings i.
Rosenheim (1913). *Biochem. J.*, **7**, 604.

References to the older literature are not given in this paper, as a complete reference to the literature on protagon and cerebrosides up to 1910 is given in the chapter by Cramer on Protagon, Cerebrosides and allied substances in the *Biochem. Handlexicon*.

LXII. NOTES ON REGULATOR MIXTURES, RECENT INDICATORS, ETC. II.

By GEORGE STANLEY WALPOLE.

From the Wellcome Physiological Research Laboratories, Herne Hill, London, S.E.

(*Received Nov. 10th, 1914.*)

In preparing the second edition of this chart I have taken the opportunity, in these notes, of attaching to it recent additions to the data which it is intended to present.

The first chart [1910] owed its origin almost entirely to a paper by Sörensen [1909, 2]: the accompanying matter was explanatory, and gave in addition a technique for applying the indicator method of Friedenthal and Salm [1907] to coloured fluids, without the use of neutral dyes.

On this occasion I have been able to avail myself of a later monograph by Sörensen [1912] and also one by Michaelis [1914]. In these are collected an invaluable store of information on the subject of indicators, solutions of standard reaction, the electrometric measurement of hydrogen ion concentration, and the properties of amphoteric electrolytes which form the foundations of so much biochemical work. In addition I have included the results of some determinations in these laboratories which are here published for the first time; and data from the general literature which are not to be found in either of the sources mentioned.

INDICATORS.

Confusion arises in descriptions of indicators and their relative practical utility because they are regarded by different authors from one or other of two essentially different points of view.

In earlier books, *e.g.* Cohn [1899] and Glaser [1901] and even in many contemporary papers indicators are described as "good" for certain titrations when their colour change occurs at a point where the reacting

substances are in chemically equivalent proportions. It is natural, therefore, that phenolphthalein should be described as "no good with ammonia," but admirable for titrating acetic acid with caustic soda.

There is, however, an alternative standard whereby an indicator may be judged, *i.e.* the fidelity with which it, by its tint, represents the $[H^{\cdot}]$ of a solution into which it is introduced.

From this standpoint these notes have been written and an indicator will be the more highly valued the less its accuracy is impaired by the presence of protein, neutral salts or other condition of practical expediency. It may be remarked in passing that phenolphthalein works perfectly well as a quantitative indicator of reaction over its sensitive range even in solutions containing ammonia. The following experiment may be cited:

20 cc. of Sörensen borate solution (NaH_2BO_3) containing phenolphthalein gave against the 0·10 N calomel electrode, with saturated KCl as connecting fluid, at 13°, a hydrogen E.M.F. 0·8630 volt ($P_H^+ = 9{\cdot}26$).

20 cc. 1 per cent. ammonium chloride solution containing the same amount of phenolphthalein +0·95 cc. N NaOH gave a liquid having the same tint. Its hydrogen E.M.F. was 0·8620 volt at 14° ($P_H^+ = 9{\cdot}21$).

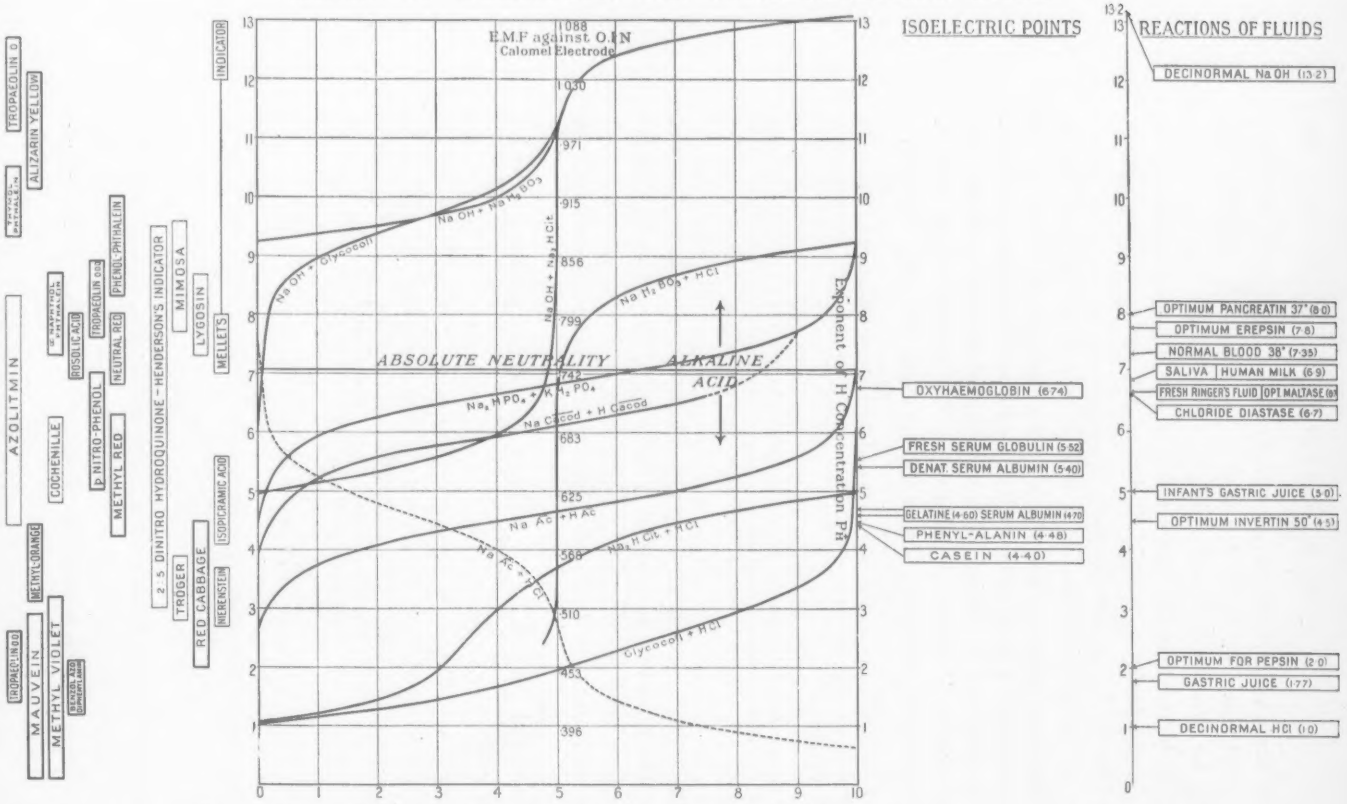
As an extreme example of those dyes which may give misleading and useless results when used as *indicators of reaction* may be mentioned *alizarin-sulphonic acid* which is even more accurate than methyl orange, when used to titrate distillates from the Kjeldahl process, especially when artificial light is used.

Adding equal quantities of it to 10 cc. each of the following Sörensen standard mixtures,

P_H^+	
7·66	(9 alk. phosphate + 1 acid phosphate) (5·26 borate + 4·74 HCl)
8·30	(9·74 alk. phosphate + 0·26 acid phosphate) (0·30 NaOH + 9·70 glycocoll) (6 borate + 4 HCl)
9·24	(1·68 NaOH + 8·32 glycocoll) (10 borate)
9·63	(2·74 NaOH + 7·26 glycocoll) (2·74 NaOH + 7·26 borate)
10·00	(4 NaOH + 6 borate),

it was seen that the dye behaved in an abnormal fashion, giving colours increasing in depth from yellow to brown, with increasing alkalinity, in the solutions containing boric acid. In the other solutions it gave purple colours

CHART OF HYDROGEN ION CONCENTRATION DATA



PARTS IN TEN OF MIXTURE OF "MORE ALKALINE" OR "LESS ACID" CONSTITUENT except in the case of the dotted curve ($NaAc + HCl$).

The Sörensen solutions are: 0·10N HCl; 0·10N NaOH; 7·505 g. glycocoll + 5·85 g. NaCl per litre; 11·876 g. $Na_2HPO_4 . 2H_2O$ per litre; 9·078 g. KH_2PO_4 per litre; 21·008 g. citric acid in 1 litre of 0·20N NaOH; 12·404 g. boric acid in 1 litre 0·10N NaOH. The other solutions are: 0·20N sodium acetate, acetic acid, sodium cacodylate, cacodylic acid. For details of plotting of dotted acetate-HCl curve, see notes.

increasing in intensity in a normal and regular fashion with the P_H^+ of the solution.

Such dyes as this, therefore, though they may be used for particular purposes under conditions thoroughly understood, merit no place in any list of approved indicators for H· concentration measurement.

Sörensen made preliminary tests of over 100 indicators but discarded so many (pyrogallol-phthalein, azo-acid-blue, chrysamine, alizarinsulphonic acid, etc.) that only 36 find places in his tabulated results. Of these he was able to recommend 20 for general use. It is significant that neither cochenille, Congo-red, alizarin nor any litmus preparation is included in the list.

In the chart, I have inserted as many as possible of these 20 approved indicators—marked specially—and, in addition, litmus (azolitmin) because it is so much used, and several others which are of interest at present in view of their recent description or for other reasons.

The list of indicators here given, when added to those in the monograph by Sörensen [1912], brings complete to the end of 1914 the whole of the indicators of which notes have been published in the available literature. In cases where a sample of the material could be obtained a test has been made of its range of sensitiveness if this had not been already done. My thanks are due to those authors who have kindly sent me specimens of their preparations. Rectangles representing the sensitive range of some of these indicators are to be found in the chart grouped immediately on the left-hand side of the curves. Where a heavy line has been used in drawing the rectangle the indicator whose name is enclosed has been approved for the colorimetric method.

2 : 5-*Dinitroquinol* [Lawrence G. Henderson and Alexander Forbes, 1910]. This indicator, obtainable from Schuchardt, is extremely useful, as it gives at a glance the approximate reaction of any solution from $P_H^+ = 3$ to $P_H^+ = 9{\cdot}5$. Over this extremely long range it shows a steady change of colour. In dilute acid it is green. If the solution be made progressively more alkaline it commences to assume a yellowish tinge when $P_H^+ = 3$, at absolute neutrality it is a bright reddish brown; when $P_H^+ = 9{\cdot}5$ a purple tint is reached which undergoes no further change with the addition of caustic alkali. The change from brown to purple is very sharp between $P_H^+ = 8{\cdot}3$ and $P_H^+ = 9{\cdot}3$: so is that from green to brown in the region $P_H^+ = 3{\cdot}4$ to $4{\cdot}7$. The convenience of having at hand an indicator working over the ranges of methyl orange, litmus and phenolphthalein will be readily appreciated.

Di-o-hydroxystyryl ketone (*Lygosin*) $CO(CH:CH.C_6H_4.OH)_2$ [Ferenz Aron, Kolosvar, 1913]. The sodium salt of this indicator is marketed by the Zimmer Factory under the name "natrium lygosinatum." For the sample supplied to me I am indebted to Prof. Aron. I found the range of its change of colour from brownish yellow to green to be exactly that of α-naphthol-phthalein ($P_H^+ = 7{\cdot}3$ to $8{\cdot}7$)—a comparative experiment with both indicators using the same regulator mixtures confirms this. If more concentrated solutions of indicator are used the colour in alkaline solution is the brown-reddish tinge described by the author. Its behaviour was examined in the series of solutions detailed on page 629. It behaved quite normally except that it was bleached, losing colour entirely after a few hours, in every series *except* those containing borate. In solutions containing borate it appeared to retain its colour indefinitely.

1-*Oxy-naphtho-chinomethane* [M. Nierenstein, Bristol. Private communication]. A sample of this indicator was kindly submitted by Dr Nierenstein for examination and its sensitive range was determined. It is colourless in solutions $P_H^+ = 2{\cdot}7$, showing a gradual increase of purple colour to $P_H^+ = 3{\cdot}7$. It is similar therefore to some of the azo indicators described by Sörensen but is of a sharper colour change than any indicator previously described which changes in this region.

6-*Sulpho-α-naphthol*-1-*azo*-m-*hydroxybenzoic acid* [M. R. Mellet, Lausanne, 1910, 1911, 1913]. The sodium salt is a violet black powder very soluble in water. In excess of alkali it is cherry red passing on the addition of acid through blue violet to bright red. Its colouring power is intense, so that quite brilliantly coloured solutions can be used without loss of accuracy in the result. The orange blue change is observed over the range $P_H^+ = 7$ to $P_H^+ = 8$: the second change, violet to red, is seen in solutions comparatively very alkaline—$P_H^+ = 12$ to $P_H^+ = 13$. I am indebted to Prof. Dr Mellet, who informs me that his indicator is still under examination, for a sample with which I have been able to confirm his earlier results.

2:6-*Dinitro*-4-*aminophenol* (*Isopicramic Acid*) [Raphael Meldola and A. J. Hale, 1912]. An alcoholic solution of this indicator is used. From the results of titrations using solutions accurately prepared gravimetrically the authors concluded that "although useless for weak acids it is possibly better than methyl orange for alkalis, of equal sensitiveness to litmus, and better than methyl orange for carbonates."

Using a sample supplied to me by Prof. Meldola I found that in solutions

more acid than $P_H^+ = 4{\cdot}1$ its colour was pink and in solutions more alkaline than $P_H^+ = 5{\cdot}6$ its colour was uniformly yellow. At intermediate reactions the changes of tint are reminiscent of methyl orange.

A sulphonic acid $C_{14}H_{15}N_4SO_3H$ [Julius Tröger and W. Hille, Brunswick, 1903]. This indicator, soluble in water, is not perfectly stable, so that a fresh solution must be prepared from time to time. Like methyl orange it is destroyed rapidly by strong alkali. It is described by the authors as being more sensitive than helianthin (methyl orange) though in its scope of application and colourations it resembles it. The sample supplied to me by the author showed the following colour changes: $P_H^+ = 2{\cdot}78$ orange pink; 2·93 ditto, but much more intense; 3·12 pale yellowish pink; 3·42 very pale brown; 3·76 yellow; 3·92 primrose yellow; 4·00 and upwards greenish primrose yellow. The range of this indicator is entered on the chart as $P_H^+ = 2{\cdot}8$ to $P_H^+ = 3{\cdot}9$. With the same regulator mixtures the methyl orange range—using two samples, one "commercial" and the other purified by repeated recrystallisation—$P_H^+ = 3{\cdot}1$ to 4·4 was confirmed. The conclusions to be drawn from my measurements are that it differs little from methyl orange in sensitiveness, and that its sensitive range is more acid than that over which methyl orange shows its colour changes.

Alizarinmonosulphonic acid [Geo. E. Knowles, 1907; compare Glaser, 1901]. This indicator, to be found amongst those described by Glaser, was discarded by Sörensen [1909, 1, 2] as being useless for the Friedenthal and Salm colorimetric method. Knowles states that it is a good substitute for methyl orange and that it can be used by artificial light. In very dilute solution I found that a sharp colour change took place between $P_H^+ = 3{\cdot}7$ (yellow) and $P_H^+ = 4{\cdot}2$ (pink). A direct comparison showed that it was even more sensitive than methyl orange over this range. Its curious behaviour with borates was noted by Knowles, and has been referred to on page 629.

3-*Amino*-2-*methylquinoline* [O. Stark, Kiel, 1907] is described as an excellent indicator which can replace methyl orange.

Compound obtained when 1 *molecule diazotised* p. *nitroaniline* + 1 *molecule of* 2-*aminonaphthol*-5: 7-*disulphonic acid is boiled with benzaldehyde, hydrochloric acid and water* [J. R. Woods, 1905]. According to the author this indicator—colourless in acid; orange in alkali—is sensitive to CO_2. This suggests a range somewhere on the alkaline side of absolute neutrality.

Dimethyl brown [Emmanuel Pozzi-Escott, 1909]. Yellow in alkaline; brown in neutral and acid solutions.

An indicator of formula $C_{11}H_8O_4$ [H. J. H. Fenton, 1906]. This indicator, and derived substances which may also be used as indicators, would appear, from the author's statements, to be of greater importance as specific reagents for amines than instruments for $H^{\cdot}$ concentration measurement.

Indicators from vegetable sources. The discovery of a valuable indicator in red cabbage and the continued use of litmus to-day suggest attention to the accounts of indicators from natural sources. Of those noted below I was able to examine the alcoholic extracts of radish skins and mimosa flowers.

Extract of red cabbage [L. E. Walbum, Copenhagen, 1913, 1, 2]. This indicator is valuable as it works accurately in the presence of proteins, neutral salts, toluene, etc. Its sensitive range is from $P_H^+ = 2$ to $P_H^+ = 4{\cdot}5$.

Radish skins [J. F. Sackur, 1910]. The radish skins were extracted with their own weight of 96 per cent. alcohol. The colour changes observed in this case in passing from normal acid to normal soda were so bewildering in number as to surpass Henderson's indicator in this respect. In addition, the colours changed rapidly although retaining their brilliance and at certain reactions showed a dichroism unequalled by any other solution I have seen not excluding eosin. The use of this extract as an indicator is attended with difficulty on account of the brilliance of these phenomena.

Mimosa flowers [Lucien Robin, 1904]. Yellow dye from mimosa flowers. Its colour change, sensitive range $P_H^+ = 7{\cdot}7$ to $9{\cdot}6$, and behaviour with borates are so strikingly reminiscent of turmeric as to suggest the identity of the colouring matter in the two cases. The two dyes tested with the solutions given on page 629 show that the colour changes of neither depend solely on the $H^{\cdot}$ concentration even when borate is not present. In the borate tubes they show brown to black colours of increasing intensity with increasing alkalinity though after some hours this colour fades but at unequal rates, leaving the greatest intensity at $P_H^+ = 9{\cdot}24$.

Iris flowers [A. Ossendowsky, 1903]. Extract of iris flowers is red in the presence of acids; green in the presence of alkalis.

Black pansy flower [E. Pozzi-Escott, 1913]. Alcoholic infusion of crushed black pansy flower: red in the presence of mineral acids passing through bluish violet with successive alkali addition till it becomes green.

Juice of the blueberry [G. N. Watson, 1913]. Alkaline—olive green: acid—rose; "sensitive to CO_2. May be used instead of cochenille or litmus."

Inorganic indicators. There may also be mentioned two "inorganic" indicators descriptions of which have been found.

Cyanogen iodide [J. H. Kastle, 1903]. Cyanogen iodide, potassium iodide and starch are recommended as very sensitive to acids.

Bismuth oxyiodide [C. Reichard, 1912]. This indicator is reported as being not sensitive to CO_2, colourless in alkaline solution and in acid yellow.

SERIES OF MIXTURES OF STANDARD REACTION.

The hydrogen E.M.F.'s of these series have been in some cases very carefully determined. Except when the curves run steep vertically the mixtures they represent are "regulator mixtures," *i.e.* they may be subjected to addition of certain quantities of acids or bases with only minimal changes in $[H^{\cdot}]$.

The seven series originally worked out by Sörensen are plotted on the chart as before. The tables connecting the composition of individual mixtures of these series, their E.M.F.'s against the 0·1 N calomel electrode, and the P_H^+ values calculated therefrom are to be found in the sources mentioned [Sörensen, 1909, 1, 2]. Similar tables referring to the curves added to the original seven in this second edition of the chart are to be found below.

The abscissae of every curve on the chart refer to the volumes in ten of the mixture of the "more alkaline" or "less acid" constituent excepting only the curve which is drawn as a dotted line. This curve refers to mixtures made up in a different fashion as explained on page 637.

Cacodylic acid-sodium cacodylate mixtures. Michaelis and Davidsohn [1912] have employed mixtures of cacodylic acid and caustic soda to obtain solutions of high "reaction inertia" which are not so acid as those obtained by mixing acids of greater dissociation constants with their salts. The solutions are not very stable. The values given in the table below are only approximate but are sufficiently accurate to indicate the range over which these mixtures can be used. They were determined by the electrometric method in these aboratories at room temperature: saturated KCl was used as connecting fluid and no correction for contact potential was made.

Composition of mixture							E.M.F.	*t.*	P_H^+
10 cc. 0·2 N cacodylic acid	+	0 cc. 0·2 N sodium cacodylate					0·5575	14	3·86
9	,,	,,	1	,,	,,	,,	0·6375	18	5·20
7	,,	,,	3	,,	,,	,,	0·6675	16	5·76
5	,,	,,	5	,,	,,	,,	0·6880	16	6·11
3	,,	,,	7	,,	,,	,,	0·7090	16	6·48
1	,,	,,	9	,,	,,	,,	?	—	?
0	,,	,,	10	,,	,,	,,	—	—	—

The solutions were made by taking successive portions of 20 cc. of a 6·9 per cent. aqueous solution of cacodylic acid (0·5 N), adding 0, 1, 3 cc. of 0·5 N NaOH and then diluting to 50 cc. in each case [compare Michaelis, 1914, p. 186].

Mixtures of acetic acid and sodium acetate,—total acetate 0·20 *normal.* Solutions of this type give hydrogen potentials which change only slightly on dilution. They may be calculated approximately from their composition by a simple formula. Results of such calculations are given by Sörensen [1912] and Michaelis [1910, 1914]. The accurate experimental results for the particular series in which the total acetate is 0·20 normal are given herewith. In the original communication [Walpole, 1914, 1, 2] are given data relating to the extent to which the hydrogen potentials of these solutions change on dilution. The correction for diffusion potential for these solutions, whether diluted or not, is negative and in no case exceeds 1·5 millivolt. It is generally much less.

Acetic acid-sodium acetate mixtures,—total acetate 0·20 *normal.*

Composition of mixture						E.M.F. (fully corrected) against 0·10 N electrode	P_H^+
0·125 cc.	0·20 N	acetic acid +	9·875 cc.	0·20 N	sodium acetate	0·7138	6·518
0·25	"	"	9·75	"	"	0·6961	6·211
0·375	"	"	9·625	"	"	0·6853	6·024
0·5	"	"	9·5	"	"	0·6778	5·894
1	"	"	9	"	"	0·6593	5·574
1·5	"	"	8·5	"	"	0·6478	5·374
2	"	"	8	"	"	0·6393	5·227
2·5	"	"	7·5	"	"	0·6316	5·093
3	"	"	7	"	"	0·6256	4·990
4	"	"	6	"	"	0·6148	4·802
5*	"	"	5	"	"	0·6046	4·626
6	"	"	4	"	"	0·5947	4·454
7	"	"	3	"	"	0·5841	4·272
8	"	"	2	"	"	0·5712	4·047
9	"	"	1	"	"	0·5525	3·723
9·25	"	"	0·75	"	"	0·5450	3·592
9·5	"	"	0·5	"	"	0·5348	3·416
9·6	"	"	0·4	"	"	0·5290	3·315
9·7	"	"	0·3	"	"	0·5225	3·202
9·75	"	"	0·25	"	"	0·5193	3·147
9·8	"	"	0·2	"	"	0·5155	3·081
9·85	"	"	0·15	"	"	0·5105	2·994
9·9	"	"	0·1	"	"	0·5057	2·912
9·95	"	"	0·05	"	"	0·4995	2·804
10	"	"	—	"	"	0·4931	2·696

The mixture marked * is "standard acetate."

Hydrochloric acid-sodium acetate series,—total acetate 0·20 *normal—total sodium* 0·20 *normal.* This series, like the one preceding, has been thoroughly worked out. It has been extended, however, so that it may be conveniently considered in two parts. The first, obtained by adding 0, 1, 2....5 cc. of N hydrochloric acid to 5 cc. N sodium acetate and making up to 25 cc. with water in each case, is the same series as the acetic-acetate series just described except that NaCl is added to each solution in amount equivalent to the acetic acid present. The result of this is to have the total sodium as well as the total acetate 0·20 normal, and each solution is for that reason just a little more acid than the corresponding solution of the series preceding. The second part is obtained by adding 5, 6, 7....10 cc. N hydrochloric acid to 5 cc. N sodium acetate and making up to 25 cc. with water in each case, and these solutions contain increasing quantities from 0 to 0·2 N of

Hydrochloric acid-sodium acetate series,—total acetate 0·20 *normal—total sodium* 0·20 *normal.*

Composition of mixture	E.M.F. (fully corrected) against 0·10 N electrode	P_H^+
0·10 cc. N HCl + 5 cc. N sodium acetate + water to 25 cc.	0·7015	6·31
0·25 " " " " "	0·6762	5·87
1 " " " " "	0·6375	5·20
1·5 " " " " "	0·6235	4·95
2 " " " " "	0·6126	4·76
2·5 " " " " "	0·6019	4·58
3 " " " " "	0·5908	4·39
3·5 " " " " "	0·5793	4·19
4 " " " " "	0·5654	3·95
4·25 " " " " "	0·5564	3·79
4·5 " " " " "	0·5461	3·61
4·625 " " " " "	0·5389	3·49
4·725 " " " " "	0·5297	3·33
4·75 " " " " "	0·5274	3·29
4·85 " " " " "	0·5159	3·09
4·875 " " " " "	0·5129	3·04
4·975 " " " " "	0·4949	2·72
5 " " " " "	0·4902	2·64
5·097 " " " " "	0·4715	2·32
5·222 " " " " "	0·4550	2·03
5·25 " " " " "	0·4525	1·99
5·35 " " " " "	0·4442	1·85
5·475 " " " " "	0·4372	1·72
5·5 " " " " "	0·4362	1·71
6 " " " " "	0·4194	1·42
6·5 " " " " "	0·4090	1·24
7 " " " " "	0·4007	1·09
8 " " " " "	0·3904	0·91
9 " " " " "	0·3812	0·75
10 " " " " "	0·3750	0·65

hydrochloric acid in addition to acetic acid and sodium chloride with respect to each of which they are all 0·2 N.

It is necessary in preparing solutions near the middle of this series to standardise the sodium acetate and the hydrochloric acid solutions against each other carefully—and an electrometric method has been devised for this purpose—and in their use to recognise that although the range of possible change of hydrogen potential under ordinary conditions is minimal the "reaction inertia" of such solutions is small. The figures below are taken from the paper referred to.

Care must be taken not to consider more or less concentrated solutions of weak acids as regulator mixtures. The addition of very little strong base leads to the freeing of so many of the anions of the weak acid that its dissociation is reversed with the consequent fall in [H], *e.g.* 1 cc. of ·01 N NaOH added to 20 cc. 0·2 N acetic acid will change the P_H^+ value by 0·11.

As the addition of strong base proceeds, however, the reaction inertia rapidly increases until an excellent regulator mixture is obtained. The shape of each of the curves is, in fact, an index of the reaction inertia of the individual members of the series of mixtures the curve represents: the tangent to the curve at any point might well be taken as the measure of the reaction inertia of the mixture represented by that point if a factor were included representing the normality of its constituents so that mixtures of different series should be comparable one with another.

ISOELECTRIC POINTS.

These data are taken from the papers of Michaelis and his collaborators. The actual figure taken in each case is that which had been obtained by the transport method.

Oxyhaemoglobin, $P_H^+ = 6{\cdot}74$ [Michaelis and Takahashi, 1910; Michaelis and Davidsohn, 1912].

Fresh serum globulin, $P_H^+ = 5{\cdot}52$ [Michaelis and Rona, 1910, 1, 2].

Denaturated serum albumin, $P_H^+ = 5{\cdot}40$ and serum albumin, $P_H^+ = 4{\cdot}7$ [Michaelis and Davidsohn, 1911, 2].

Gelatin, $P_H^+ = 4{\cdot}60$ [Michaelis and Grineff, 1912].

Caseinogen, $P_H^+ = 4{\cdot}4$ [Michaelis and Rona, 1910, 1, 2; Michaelis and Pechstein, 1912].

Phenylalanine, $P_H^+ = 4{\cdot}48$ [Michaelis, 1912].

OPTIMUM REACTIONS FOR ENZYMES.

These data, and much accompanying and explanatory matter, are for the most part collected in Michaelis' monograph [1914]. See also Sörensen [1912]. They do not all appear on the chart for lack of space.

The following references are to original papers.

Tryptic enzymes [Kurt Meyer, 1911; Palitsch and Walbum, 1912; Michaelis and Davidsohn, 1911, 1].

Invertin [Sörensen, 1909, 1, 2; Michaelis and Davidsohn, 1911, 3].

Peptic and Ereptic enzymes [Rona and Arnheim, 1913; Sörensen, 1909, 1, 2; Michaelis and Davidsohn, 1911, 4].

Lipase [Davidsohn, 1912, 1, 2; Rona and Bien, 1914, 1, 2].

Catalase [Sörensen, 1909, 1, 2; Michaelis and Pechstein, 1913; Waentig, Percy, and Steche, 1911].

Maltase [Michaelis and Rona, 1913, 1, 2].

Diastase [Michaelis and Pechstein, 1914; Norris, 1913, 1, 2].

REACTIONS OF PHYSIOLOGICAL FLUIDS.

Saliva [Michaelis and Pechstein, 1914].

Gastric Juice [Fränkel, 1905; Michaelis and Davidsohn, 1910].

Infants' Gastric Juice [Allaria, 1908; Davidsohn, 1912, 2].

Intestinal Juice [Auerbach and Pick, 1912].

Blood [Hasselbalch and Lundsgaard, 1912].

Human Milk [Davidsohn, 1913].

REFERENCES.

Allaria (1908). *Jahrb. Kinderheilk.*, **67**, 123.
Aron (1913). *Pharm. Post*, **46**, 521.
Auerbach and Pick (1912). *Arbeiten aus dem kaiserl. Gesundheitsamt*, Berlin, **43**, 155.
Cohn (1899). *Indicators and Test Papers.* Chapman and Hall, London.
Davidsohn (1912, 1). *Biochem. Zeitsch.*, **45**, 284.
—— (1912, 2). *Zeitsch. Kinderheilk.*, **4**, 208.
—— (1913). *Zeitsch. Kinderheilk.*, **9**, 11.
Fenton (1906). *Proc. Camb. Phil. Soc.*, **3**, 298.
Fränkel (1905). *Zeitsch. exp. Pathol. Therapie*, **1**, 431.

Friedenthal and Salm (1907). *Zeitsch. Elektrochem.*, **13**, 125.
Glaser (1901). *Indikatoren der Acidimetrie u. Alkalimetrie.* Kriedel, Wiesbaden.
Hasselbalch and Lundsgaard (1912). *Biochem. Zeitsch.*, **38**, 77.
Henderson and Forbes (1910). *J. Amer. Chem. Soc.*, **32**, 687.
Kastle (1903). *Amer. Chem. J.*, **30**, 87,
Knowles (1907). *J. Soc. Dyers*, **23**, 120.
Meldola and Hale (1912). *Chemical World*, **1**, 327.
Mellet (1910). *Chem. Zeit.*, **34**, 1073.
—— (1911). *Moniteur scientifique*, **75**, 576.
—— (1913). *Chem. Zeit.*, **37**, 666.
Meyer (1911). *Biochem. Zeitsch.*, **32**, 274.
Michaelis (1910). *Handbuch der biochemischen Arbeitsmethoden*, **3**. Urban and Schwarzenberg, Berlin.
—— (1912). *Biochem. Zeitsch.*, **47**, 251.
—— (1914). *Die Wasserstoffionen-Konzentration.* Julius Springer, Berlin.
Michaelis and Davidsohn (1910). *Zeitsch. exp. Pathol. Therapie*, **8**, 2.
—— (1911, 1). *Biochem. Zeitsch.*, **30**, 481.
—— (1911, 2). *Biochem. Zeitsch.*, **33**, 456.
—— (1911, 3). *Biochem. Zeitsch.*, **35**, 386.
—— (1911, 4). *Biochem. Zeitsch.*, **36**, 280.
—— (1912). *Biochem. Zeitsch.*, **41**, 102.
Michaelis and Grineff (1912). *Biochem. Zeitsch.*, **41**, 373.
Michaelis and Pechstein (1912). *Biochem. Zeitsch.*, **47**, 260.
—— (1913). *Biochem. Zeitsch.*, **53**, 320.
—— (1914). *Biochem. Zeitsch.*, **59**, 77.
Michaelis and Rona (1910, 1). *Biochem. Zeitsch.*, **27**, 38.
—— (1910, 2). *Biochem. Zeitsch.*, **28**, 193.
—— (1913, 1). *Biochem. Zeitsch.*, **57**, 70.
—— (1913, 2). *Biochem. Zeitsch.*, **58**, 148.
Michaelis and Takahashi (1910). *Biochem. Zeitsch.*, **29**, 439.
Norris (1913, 1). *Biochem. J.*, **7**, 26.
—— (1913, 2). *Biochem. J.*, **7**, 622.
Ossendowsky (1903). *J. Russ. Phys. Chem. Soc.*, **35**, 845.
Palitsch and Walbum (1912). *Biochem. Zeitsch.*, **47**, 1.
Pozzi-Escott (1909). *Bull. Assoc. Chim. Sucr. Dist.*, **27**, 560.
—— (1913). *Ann. Chim. Anal.*, **18**, 58.
Reichard (1912). *Pharm. Zentr.*, **53**, 1033.
Robin (1904). *Ann. Chim. Anal.*, **9**, 130.
Rona and Arnheim (1913). *Biochem. Zeitsch.*, **57**, 84.
Rona and Bien (1914, 1). *Biochem. Zeitsch.*, **59**, 100.
—— (1914, 2). *Biochem. Zeitsch.*, **64**, 13.
Sackur (1910). *Chem. Zeit.*, **34**, 1192.
Sörensen (1909, 1). *Biochem. Zeitsch.*, **21**, 131.
—— (1909, 2). *Compt. rend. Lab. Carlsberg*, **7**, 1.
—— (1912). *Ergebn. der Physiol.*, **12**, 393.
Stark (1907). *Ber.*, **40**, 3434.
Tröger and Hille (1903). *J. pr. Chem.*, **68**, 297.
Waentig, Percy and Steche (1911). *Zeitsch. physiol. Chem.*, **72**, 226.
Walbum (1913, 1). *Biochem. Zeitsch.*, **48**, 29.
—— (1913, 2). *Compt. rend. Lab. Carlsberg*, **10**, 227.
Walpole (1910). *Biochem. J.*, **5**, 207.
—— (1914, 1). *J. Chem. Soc.*, **106**, 2501.
—— (1914, 2). *J. Chem. Soc.*, **106**, 2521.
Watson (1913). *Amer. J. Pharm.*, **85**, 246.
Woods (1905). *J. Soc. Chem. Ind.*, **24**, 1284.

LXIII. THE ESTIMATION OF ALLANTOIN IN URINE IN THE PRESENCE OF GLUCOSE.

By ROBERT HENRY ADERS PLIMMER
and RUTH FILBY SKELTON.

From the Ludwig Mond Research Laboratory for Biological Chemistry, Institute of Physiology, University College, London.

(*Received Nov. 13th, 1914.*)

It has been shown by us in a previous communication [1914] that the urea and allantoin in urine can be estimated by Folin's magnesium chloride method; the urea is estimated by means of urease and the difference between the two values is the amount of allantoin present. Glucose in the urine does not affect the determination of urea by means of urease, as shown by Marshall [1913], but it causes a loss of from 9–30 per cent. of the nitrogen of the urea when Folin's magnesium chloride method is used, as shown by Mörner [1903]. The humin which is formed by heating the glucose in urine with hydrochloric acid contains nitrogen [Udransky, 1888]. The same error applies to the estimation of allantoin. Mörner overcame the difficulty in the case of urea by precipitating the glucose with baryta mixture, alcohol and ether, and estimating the urea in the filtrate, but this procedure does not serve for the estimation of allantoin, since it is precipitated with the glucose [Mörner, 1903; Haskins, 1906].

Wiechowski's method for the estimation of allantoin in urine depends upon the precipitation of allantoin as mercury compound after pigments, chlorides and other compounds have been removed, and is not affected by the presence of glucose, but for this method large amounts of urine are required and the estimation takes several days to perform.

The Folin method if it could be adapted to the estimation of allantoin would be of advantage, in that only small quantities of urine are required, which is important when other estimations, *e.g.* of "acetone bodies," have to be performed, and it would be of further advantage in that the results are quickly obtained.

In order to make use of the Folin method it is essential that the glucose be removed from solution. This removal of glucose was found to be a more troublesome process than was anticipated. Several methods were attempted before it was found that the glucose could be completely precipitated by basic lead acetate and sodium hydroxide.

(1) *Removal of Glucose by Fermentation.*

The simplest way of removing glucose seemed to be by fermentation by yeast. Mörner had already tried this method and found it not to be entirely satisfactory. Some preliminary experiments indicated that this method might be possible. The estimation of urea and allantoin was carried out on 5 cc. of urine, and on 5 cc. of urine to which 5 cc. of a 5 per cent. solution of glucose had been added and removed by fermentation. Our data in cc. of 0·1 N ammonia were:

Urine		Urine + Glucose		Urine		Urine + Glucose	
15·0	15·15	15·3	15·15	49·4	49·7	49·7	49·55
15·3		15·0		50·0		49·6	

Further experiments did not give such satisfactory results:

16·2	16·25	15·9	15·7	53·2	53·15	52·2	51·9
16·3		15·3		53·1		51·6	

It was possible that in these experiments the conditions for the fermentation were not suitable, and it seemed to us that if the conditions for complete fermentation could be found the removal of glucose by the action of yeast would be possible. Numerous experiments were made to test the rate of fermentation of glucose by yeast. 10 cc. of 1, 2, and 3 per cent. solutions of glucose in water were fermented with 1 to 10 cc. of 1 to 5 per cent. suspensions of yeast in water for periods of 6 to 20 hours; the solutions were filtered and the filtrates tested for glucose. The results were extremely variable, as is seen in the following summary:

Series of expts.	Per cent. of glucose solution	Per cent. of yeast suspension added	Number of cc. of yeast suspension required to ferment the glucose	Time of fermentation in hours
1	1	3	4	6
2	1	5	3	6
3	2	1	5	16
4	2	3	4	16
5	2	5	7	6
6	2	5*	5	17
7	2	5	7	17
8	3	3*	6	17
9	3	3	10	20
10	3	4*	6	17
11	3	4	6	20
12	3	5	6	20
13	3	5	10	17

* Small quantities of glucose still left unfermented.

The variability which is particularly noticeable in experiments 3 and 6, and 5 and 7, is mainly accounted for by the use of different samples of yeast, but even when the same sample of yeast was used and the fermentation carried out with the same quantity of glucose dissolved in urine the glucose was not always completely removed in the expected time.

Not only was the rate of fermentation unreliable, probably due to variations in the acidity of the urine, but also it was found necessary to make a correction for the amount of nitrogen introduced into the solution with the yeast.

Though the desired results can often be obtained, there is so much uncertainty in the removal of glucose by fermentation that the method is not a suitable one for practical use.

Some experiments were also made with zymin and with yeast dried according to v. Lebedeff's method and kindly given to us by Prof. Harden. The fermentation of the glucose was much slower, and a still greater correction for the amount of nitrogen introduced in the preparation had to be made.

(2) *Removal of Glucose by Benzoylation.*

Glucose and other carbohydrates give insoluble benzoyl esters when they are acylated by the Schotten-Baumann method. This method was therefore tested after adding glucose to urine and shaking the solution with varying quantities and excess of benzoyl chloride and sodium hydrate. In only one experiment out of 11 was the glucose completely removed. Further in the strongly alkaline liquid there is loss of ammonia from the urine. This method was therefore not serviceable.

(3) *Removal of Glucose by Precipitation with Cupric Hydroxide.*

Salkowski [1872, 1879] observed that glucose was completely precipitated in combination with cupric hydroxide when five molecules of copper sulphate and eleven molecules of sodium hydroxide were added for every molecule of glucose; Yoshimoto [1908] repeated these experiments and extended them to the precipitation of other carbohydrates. We can confirm the results with glucose; the glucose is completely precipitated if the proportions are exactly those given by Salkowski but not otherwise. A slight excess of alkali dissolves the copper hydroxide compound.

Owing to the sensitiveness of the precipitation towards alkali it was not anticipated that its application to the removal of glucose from urine of varying acidity would give the desired result. The precipitate with urine is of large bulk, the filtration is slow and the alkalinity of the solution leads to loss of ammonia during filtration. Glucose was generally found to be present in the filtrate. This method is again not applicable to urine.

(4) *Removal of Glucose with Lead Hydroxide.*

Glucose is not precipitated from solution by lead acetate; it is partially precipitated by basic lead acetate. It is also stated that glucose is removed from solution by basic lead acetate and ammonia, when these reagents are added to diabetic urine in the proportions used in the estimation of aceto-acetic acid and hydroxybutyric acid by Shaffer and Marriott's method [1913]. We have tested urine, to which glucose had been added, for glucose after such a precipitation and have found it still present, so that its precipitation under these conditions is not complete.

It would no doubt be possible to find the exact proportions of these reagents which would completely precipitate the glucose, but since for the purpose of estimating allantoin ammonia cannot be used we have used sodium hydroxide instead. Our experience with copper hydroxide suggested that the precipitation of glucose would be complete if the proportions were:

$$2C_6H_{12}O_6 : 5\,(Pb\,(OH)_2\,.\,Pb\overline{ac}_2) : 10NaOH,$$

and on testing these proportions it was found that the precipitation of glucose was complete. The filtrate from the precipitate shows no reduction or, in a few cases only, a very slight reduction of Fehling's solution.

This precipitation with lead hydroxide is not so sensitive to excess of sodium hydroxide as that with copper hydroxide. A slight excess of sodium hydroxide may be added, and in many experiments as much as 2·5 molecules

of sodium hydroxide were used, and the whole of the glucose removed. A larger excess of the sodium hydroxide leads to solution of the precipitate.

This method can therefore be applied to the estimation of allantoin in urine containing glucose.

The Effect of Basic Lead Acetate and Sodium Hydroxide upon Allantoin.

The estimation of allantoin in urine by the Folin method will be therefore possible if allantoin is not precipitated by the basic lead acetate and sodium hydroxide, or by the glucose lead hydroxide compound. Two series of experiments were therefore made to test if allantoin were so precipitated.

A. 25 cc. of approximately 0·1 N allantoin solution were treated with the amounts of basic lead acetate and sodium hydroxide required assuming that the solution contained 1, 2, 3 and 4 per cent. of glucose.

B. 25 cc. of the same allantoin solution were treated with the same quantities of basic lead acetate and sodium hydroxide in the presence of 1, 2, 3 and 4 per cent. of glucose.

In both cases the volumes were made up to 250 cc., the precipitates filtered off and the nitrogen estimated by Kjeldahl's method in 50 cc. (= 5 cc. of the original solution) of the filtrate. A total nitrogen estimation, C, was also made in 5 cc. of the original solution.

	A. cc. 0·1N NH_3	B. cc. 0·1N NH_3	C. cc. 0·1N NH_3
1 per cent.	2·6	—	2·7
2 „	2·6	2·2	
3 „	2·5	2·2	
4 „	2·5	2·2	
1 per cent.	2·5	2·45	2·75
2 „	2·35	2·3	
3 „	2·5	2·5	
4 „	2·45	2·4	

Though the amount of nitrogen in the filtrate was in all cases less than in the original solution, it cannot be concluded that allantoin is precipitated by basic lead acetate and sodium hydroxide. It is most probable that this difference is due to the precipitation of traces of impurity, such as uric acid, in the allantoin. It is unlikely that the same figure would have been obtained in the series of experiments in which quantities of basic lead acetate and sodium hydroxide varying from 14 to 56 cc. and 4 to 17 cc. respectively were used.

Application of the Method to Urine.

In applying this precipitation of glucose by basic lead acetate and sodium hydroxide to urine the ammonia present in the urine must be removed, as the reagent is alkaline and the ammonia is slowly evolved during filtration. The removal of ammonia presents no difficulty if it be effected by Folin's air current method. The solution which remains can be used for the allantoin estimation, the excess of sodium carbonate being neutralised with acetic acid, using phenolphthalein as indicator, and warming to remove the carbon dioxide before the precipitation of the glucose. Owing to the presence of phosphates, sulphates, pigments and other compounds in urine an excess of basic lead acetate above that required for the amount of glucose present must be added: 25 cc. of basic lead acetate and the corresponding amount of sodium hydroxide have been found to suffice for 25 cc. of dog's urine (when the 24 hours quantity has been diluted to 500 cc.).

In performing the hydrolysis by the magnesium chloride method with large quantities of liquid the water which distils into the special form of condenser is emptied out as required till the concentration is sufficient for the hydrolysis of the urea and allantoin.

The experimental details are as follows:

25 cc. of urine are treated with 1 gram of sodium carbonate and the ammonia estimated by Folin's method; it is not necessary to use paraffin or toluene to prevent frothing if tall cylinders are employed. The solution is washed into a 250 cc. measuring flask, and the sodium carbonate neutralised by carefully adding glacial acetic acid from a burette and warming. To the neutral solution 25 cc. of basic lead acetate + 14 cc. of basic lead acetate for every per cent. of glucose are added. These quantities require 6·2 + 3·4 cc. of 2N sodium hydroxide to precipitate the glucose and to remove any excess of lead acetate. It is advisable to determine the exact amount of 2N sodium hydroxide which is required to precipitate the lead hydroxide from the basic lead acetate by a special experiment, as in making up solutions of the basic lead acetate and 2N sodium hydroxide the solutions have never quite the same concentration.

The caustic soda is added slowly from a burette with constant shaking; the solution is made up to 250 cc. and the precipitate filtered off.

50 cc. of the filtrate should be tested for glucose before proceeding to the allantoin estimation, as it occasionally happens that the glucose is not completely removed. A slight reduction has no effect upon the result. If there

is considerable reduction the removal of the glucose must be repeated in another sample. We are unable to explain why the precipitation sometimes fails, but it seems most probable that it is due to improper neutralisation of the sodium carbonate.

50 cc. of the filtrate (= 5 cc. of urine) are used for the estimation of the urea + allantoin by Folin's magnesium chloride method.

We have tested the method upon urine to which glucose had been added with the following results, a control (= NH_3 + urea + allantoin) having been previously made; the figures are in cc. of 0·1 N ammonia:

Glucose added %	Reduction of filtrate	Urea + allantoin	NH_3 per 5 cc.	NH_3 + urea + allantoin	Difference
1·3	0	42·7	3·0	45·8	−0·1
2·0	+	—	3·0	45·8	—
3·0	0	42·4	3·0	45·8	−0·4
4·0	0	42·9	3·0	45·8	+0·1
1	+	—	1·2	36·2	—
2	slight	35·0	1·2	36·2	0
3	0	35·1	1·2	36·2	+0·1
4	0	34·5	1·2	36·2	−0·5
1·2	very slight	32·3	2·0	34·5	−0·2
2	,,	32·5	2·0	34·5	0
3	0	32·6	2·0	34·5	+0·1
4	0	32·8	2·0	34·5	+0·3
1	very slight	32·4	2·0	34·5	−0·1
2	0	32·0	2·0	34·5	−0·5
3	0	32·3	2·0	34·5	−0·2
4	slight	32·6	2·0	34·5	+0·1
1	very slight	32·5	2·0	34·5	0
2	,,	32·4	2·0	34·5	−0·1
4	,,	32·6	2·0	34·5	+0·1
5	0	32·5	2·0	34·5	0
1	+	—	2·1	39·3	—
2	0	36·8	2·1	39·3	−0·4
3	0	37·0	2·1	39·3	−0·2
4	0	36·8	2·1	39·3	−0·4

The maximum difference is − 0·5 cc., but in many of the experiments there was no difference: a difference of 0·4 cc. is often obtained in two parallel experiments with normal urine; it cannot be regarded as a great discrepancy, as the Folin method is a difficult one to perform.

Analyses of Diabetic Dog's Urine.

We have devised this method for the analysis of diabetic dog's urine and we have been fortunate in being able to use it upon the urine of a dog rendered diabetic by Prof. Starling. The analyses have been performed by

Mr W. W. Reeve, who has carried them out according to our directions. No difficulty was experienced in the removal of the glucose, though the solutions sometimes showed a slight reduction. The output of allantoin of a diabetic dog is greater than that of a normal dog upon a similar diet. The data in cc. 0·1 N ammonia per 5 cc. urine are the following:

Amount of glucose present in %	NH_3	NH_3 + urea	Urea + allantoin	Allantoin
1·0	1·2	29·1	28·8	0·9
5·6	1·4	30·8	31·9	2·5
1·2	0·9	10·8	12·3	2·4
4·5	1·5	28·5	29·0	2·0
4·0	1·5	24·4	25·2	2·3
3·0	2·4	34·1	35·8	4·1
5·8	2·1	35·9	36·4	2·6
3·8	2·9	37·6	38·4	3·7
6·0	2·2	35·7	36·4	2·9
3·0	2·4	32·5	33·7	3·6
3·5	2·9	35·9	36·7	3·7

The fact that these analyses have been made by an independent worker shows that the method is satisfactory.

Summary.

The estimation of allantoin in urine containing glucose can be effected by Folin's magnesium chloride method if the glucose be first removed. The glucose can be removed by precipitation with basic lead acetate and sodium hydroxide if the proportions are $2C_6H_{12}O_6 : 5\,[Pb(OH)_2 . Pb\overline{ac}_2] : 10NaOH$.

The expenses of this research and of the previous one upon the estimation of urea and allantoin have been defrayed by a grant to one of us from the Government Grant Committee of the Royal Society, to whom we desire to express our thanks.

References.

Haskins (1906). *J. Biol. Chem.*, **2**, 243.
Marshall (1913). *J. Biol. Chem.*, **14**, 283.
Mörner (1903). *Skand. Arch. Physiol.*, **14**, 297.
Plimmer and Skelton (1914). *Biochem. J.*, **8**, 70.
Salkowski (1872). *Pflüger's Arch.*, **5**, 220.
—— (1879). *Zeitsch. physiol. Chem.*, **3**, 79.
Shaffer and Marriott (1913). *J. Biol. Chem.*, **16**, 265.
Udransky (1888). *Zeitsch. physiol. Chem.*, **12**, 42.
Yoshimoto (1908). *Zeitsch. physiol. Chem.*, **56**, 425.

LXIV. THE GRAVIMETRIC ESTIMATION OF MINUTE QUANTITIES OF PHOSPHORUS.

By HENRY STANLEY RAPER.

From the Department of Physiology, the University of Leeds.

(*Received Nov. 14th, 1914.*)

In the course of an investigation concerning certain of the phenomena of immunity, a method was required for the estimation of the ether-soluble phosphorus compounds of blood serum. The amount of serum available for the estimation was not more than 10 cc. and it was anticipated that only a few milligrams of the phosphorus compounds in question would be present. The isolation of this small amount and its direct weighing was therefore rejected as a possible method of estimation. An estimation of the phosphorus present appeared to be much more promising, since Neumann's method was known to be accurate for quantities of P_2O_5 as low as 10 to 15 milligrams. It was thought that by suitable modification (the use of decinormal instead of half normal solutions) this process could be used for smaller quantities of phosphorus. For the purpose of working out the method a standard solution of potassium dihydrogen phosphate solution was made such that 5 cc. contained 1 milligram P_2O_5. Addition to this of 10 cc. of the acid mixture (equal parts of nitric and sulphuric acids), and subsequent dilution to about 100 cc. made a solution comparable to one which would be obtained by the Neumann method after oxidation had been completed and the acid residue diluted. A solution prepared in this way was therefore used in all experiments when it was desired to test the applicability of the method to actual working conditions with an unknown substance. In order to obtain rapid and complete precipitation of the phosphorus as ammonium phosphomolybdate it has been found most satisfactory to neutralise a portion of the acid present with ammonia, to add ammonium nitrate and then precipitate with ammonium molybdate at 80°. The titration of this precipitate by the Neumann method using decinormal solutions was found to

be unsatisfactory. The results were from 10 to 20 per cent. too high, when 1 milligram of P_2O_5 was being estimated. On this account the method was rejected as unsuitable, a method with an error of less than 5 per cent. being required.

The next procedure tried was the lead molybdate method of Brearley and Ibbotson [1902], which was devised by them for the estimation of phosphorus in steel. The principle of the method is very ingenious. The ammonium phosphomolybdate precipitate is dissolved in ammonia and the molybdic acid in it is precipitated as lead molybdate, which is capable of being ignited and weighed. This lead molybdate precipitate weighs 142 times as much as the phosphorus originally taken, so that even a tenth of a milligram of phosphorus gives rise to a weighable amount of precipitate. On applying this method to the precipitate of phosphomolybdate obtained in the Neumann method, the results were constantly between 10 and 12 per cent. too high when 1 milligram of P_2O_5 was being estimated. It was found, however, that if the phosphorus was precipitated by ammonium molybdate from a solution containing only ammonium nitrate and nitric acid, no sulphuric acid being present, the lead molybdate method gave very accurate results. The presence of sulphuric acid thus appears to cause precipitation of a small but appreciable quantity of molybdic oxide along with the ammonium phosphomolybdate, and although this causes no marked error when upwards of 20 milligrams of P_2O_5 are being estimated by the Neumann method, the error is magnified when only 1 milligram of P_2O_5 is in question. To overcome this difficulty the ammonium phosphomolybdate precipitate was dissolved in ammonia and reprecipitated in the presence of nitric acid and ammonium nitrate. This proved quite satisfactory and yielded results in which the average error with 1 milligram of P_2O_5 was only about 2 per cent. It was discovered later that the above influence of sulphuric acid in the estimation of phosphorus by the phosphomolybdate method had been observed by Hibbard [1913]. This author also recommends reprecipitation to overcome the difficulty.

Certain other interesting facts have been observed in working out the method finally adopted.

In determining the best amounts of ammonium nitrate and nitric acid to use in reprecipitating the ammonium phosphomolybdate, it was found that although ammonium nitrate in considerable amounts favoured rapid precipitation, the results were always from 6 to 10 per cent. too high unless the amount of nitric acid was largely increased. This in its turn rendered

precipitation less rapid and incomplete. The effect of the ammonium nitrate is due to the ease with which it causes precipitation of molybdic oxide when insufficient nitric acid is present. A determination of the ideal amounts of ammonium nitrate and nitric acid required to produce complete precipitation of 1 milligram of P_2O_5 in 100 cc. of liquid within 20 minutes without concurrent precipitation of molybdic oxide was therefore made; these concentrations were then adhered to in the reprecipitation of the phosphomolybdate.

In addition, it was observed that the use of old ammonium molybdate solution in which a white crystalline deposit had appeared, gave rise to high results. On examination it was shown that such solutions gave rise to precipitation of molybdic oxide in the presence of amounts of nitric acid and ammonium nitrate which did not cause precipitation of molybdic oxide when fresh molybdate solutions were used.

Whilst this work was in progress two methods for the estimation of small quantities of phosphorus were published by Taylor and Miller [1914]. The first method is a modification of the Neumann titration method, and the second is a colorimetric method. In both cases the substance analysed is ashed in the dry condition and the phosphorus subsequently precipitated as ammonium phosphomolybdate. The first method is stated to be accurate to 2 per cent. with amounts of P_2O_5 as low as 1 milligram. The second method is less accurate but can be used for smaller amounts than the first. Both appear to be more cumbersome than the one described in the present paper. Further, it has been found possible by the dry ashing method (which is always preferable to the wet method if it can be adopted), followed by precipitation as ammonium phosphomolybdate and then as lead molybdate, to estimate 0·1 milligram of phosphorus with an error of only 3 per cent. In the following description of the method, the process of precipitating the lead molybdate is given almost exactly as described by Brearley and Ibbotson. An alternative method suggested by them, in which the precipitate contains the phosphorus as lead phosphate in addition to the lead molybdate, has not been found as accurate.

Solutions required.

1. Ammonium Nitrate 50 per cent.
2. Ammonium Acetate 50 per cent.
3. Ammonium Chloride 20 per cent.
4. Lead Acetate 4 per cent.
5. Ammonium Molybdate 10 per cent. (To be made with cold water.)
6. Standard solution of KH_2PO_4; 0·3833 grams per litre; 5 cc. = 1 milligram P_2O_5.

The substance is oxidised in the usual fashion with nitric and sulphuric acids in a Kjeldahl flask. With the small amounts of substance used in the present investigation, rarely more than 10 cc. of the acid mixture were required. The volume of acid left after the oxidation is estimated by pouring water from a measuring cylinder into a similar Kjeldahl flask. The contents of the flask are rinsed into a 500 cc. conical flask which has been marked at the 100 cc. level. 15 cc. of ammonium nitrate are added, and for every cc. of acid left after the oxidation, 0·75 cc. of strong ammonia (S.G. 0·880) in addition. The solution is diluted till the volume is about 100 cc. and is then heated to 80°. 10 cc. of ammonium molybdate are added and the contents of the flask are constantly agitated for about a minute and a half. If precipitation of the phosphomolybdate has not begun, less than one milligram of P_2O_5 is present and 5 cc. of the standard phosphate solution should be added, or precipitation will not be completed quickly enough. The contents of the flask are again shaken, then maintained at 80° for 20 minutes, shaking twice during this period, each time for a minute. The precipitated ammonium phosphomolybdate is filtered off by means of suction on a pulp filter made by shaking a 9 cm. Swedish filter paper with very dilute nitric acid (about 2 per cent.), and pouring the pulp a little at a time on to a 2 cm. perforated porcelain plate in a funnel of suitable size. A 3 inch funnel has been found most convenient. The pulp is well packed down, employing suction by means of a Buchner flask, and is finally pressed down and at the sides with the finger. Prepared in this way the filter always retains the phosphomolybdate precipitate. The flask is rinsed out twice with cold water and the precipitate washed twice on the pulp with cold water using a wash bottle with the jet removed. The precipitate is now dissolved in 6 cc. of dilute ammonia (10 per cent.), which is allowed to filter through into the rinsed-out precipitation flask. The pulp is washed about half a dozen times with water, as much as possible being removed from the pulp each time by suction. For this purpose the stem of the funnel is passed through a rubber stopper bored with two holes. Through the second hole a bent tube passes which can be connected to the pump. The filtrate and washings, which should measure rather less than 100 cc., are now acidified by adding 11 cc. of concentrated nitric acid, and 8 cc. ammonium nitrate added. The liquid is heated to 80° and the phosphorus again precipitated as phosphomolybdate by adding 7 to 8 cc. of ammonium molybdate. The flask is well shaken for from one to two minutes and then allowed to stand at 80° for 20 minutes, with occasional shaking. The precipitate is filtered off on

a pulp filter, prepared as before, washed thoroughly with water and dissolved in 6 cc. of dilute ammonia, the pulp being then washed several times with water. The filtrate and washings are best collected in the washed-out precipitation flask. They are transferred to a beaker, 11 cc. concentrated hydrochloric acid added followed by 10 cc. of lead acetate, and the contents then heated almost to boiling. Meanwhile in the precipitation flask, a mixture of 50 cc. of ammonium chloride and 50 cc. of ammonium acetate is heated to boiling point. When both solutions are hot the solution in the beaker is poured into that in the flask, the beaker is rinsed out with a little hot water, and the precipitation of the lead molybdate facilitated by shaking. The precipitate is filtered off on a Gooch crucible, well washed with hot water, dried, ignited and weighed. The weight of the precipitate in milligrams multiplied by 0·0069 gives the weight of phosphorus in milligrams. The factor 0·0159 gives the weight of P_2O_5. The factors theoretically are 0·00703 and 0·0161. Since the results are on an average 2 per cent. too high, the amended factors have been used when reprecipitation was necessary. When this is not the case the theoretical factors may be used. If 5 cc. of the standard phosphate solution have been added in the first precipitation, then 1 milligram P_2O_5 or 0·437 milligram of phosphorus must be subtracted from the weight found.

The method just outlined has been used for the estimation of the ether-soluble phosphorus compounds of serum. The dry ashing method for the preliminary oxidation was not convenient, because of the large volume of the ether solution of the phosphorus compounds, and the difficulty of evaporating this in a small platinum dish without loss. In cases where the dry ashing method can be used, however, the reprecipitation of the ammonium phosphomolybdate is not necessary. The method of ashing adopted by Taylor and Miller [1914] may be used with advantage. The substance, to which a few drops of saturated sodium carbonate solution have been added, is dried in a platinum dish and ignited. The mass is dissolved in a solution of 8·5 per cent. nitric acid containing 6·5 per cent. ammonium nitrate and after precipitation with ammonium molybdate the phosphorus is determined by the lead molybdate method. It is necessary to know approximately the amount of phosphorus present. For each tenth of a milligram of phosphorus about 25 cc. of the ammonium nitrate-nitric acid mixture must be used for dissolving the mass after ignition and rinsing out the platinum dish. The solutions used in the precipitation of the lead molybdate must be taken in about the same relative proportion, *i.e.* 25 cc. of the ammonium chloride

and ammonium acetate mixture, 2·5 cc. of concentrated hydrochloric acid and 2·5 cc. of 4 per cent. lead acetate solution. When about one to two tenths of a milligram of phosphorus is being estimated it is preferable to use a 100 cc. conical flask, a 1 cm. filter plate and a smaller amount of pulp in the filtration. Usually about a quarter of a 9 cm. Swedish filter paper has been found to be sufficient.

Special emphasis must be laid on the necessity for adhering to the relative concentrations of ammonium nitrate and nitric acid given above, when the phosphoric acid is being precipitated as ammonium phosphomolybdate. It is only in this way that a concurrent precipitation of molybdic oxide is avoided, and much time was lost in the early stages of working out the method through this fact not being appreciated. When Neumann's method of oxidation is used and reprecipitation of the phosphomolybdate is necessary, a trace of molybdic acid in the precipitate appears to be unavoidable, probably due to traces of colloidal matter from the filter pulp facilitating its precipitation. This error has been corrected by a slight reduction in the factor finally used for converting the weight of lead molybdate obtained into the corresponding weight of phosphorus.

The method has so far been used only for the purpose for which it was devised, but there is little doubt that it would serve equally well for the quantitative estimation of phosphorus in most organic substances.

Appended are some of the results obtained by the method both with known and unknown amounts of phosphorus:

Estimation of Known Amounts of Phosphorus.

Single Precipitation Method.			*Reprecipitation Method.*		
Phosphorus taken in milligrams	Phosphorus found	Mean	Phosphorus taken in milligrams	Phosphorus found	Mean
	0·436			0·451	
0·437	0·443	0·439	0·437	0·435	0·445
	0·443			0·453	
	0·434			0·454	
0·175	0·179			0·431	
0·089	0·094			0·448	
	0·091				

Using the factor 0·0069 instead of the theoretical factor 0·007 the mean value becomes 0·437.

Estimation of Unknown Amounts of Phosphorus (Reprecipitation Method).

	Phosphorus found, milligrams		Phosphorus found, milligrams
Solution of crude lecithin in ether 10 cc.	0·66	Ether-soluble phosphorus compounds from 10 cc. ox serum	0·68
	0·66		0·68
	0·66		
5 cc.	0·33		
2 cc.	0·14		
	0·15		

SUMMARY.

A method is described for the gravimetric estimation of fractions of a milligram of phosphorus as low as 0·1 milligram. It is a modification of Brearley and Ibbotson's method for the estimation of phosphorus in steel.

After preliminary oxidation to phosphoric acid, the phosphorus is precipitated under special conditions as ammonium phosphomolybdate. The molybdenum in this precipitate is then estimated as lead molybdate.

The method is rapid and accurate and requires no expensive or special apparatus.

REFERENCES.

Brearley and Ibbotson (1902). *Analysis of Steel Works Materials*, p. 55 (Longmans).
Hibbard (1913). *J. Ind. Eng. Chem.*, **5**, 998.
Taylor and Miller (1914). *J. Biol. Chem.*, **18**, 216.

LXV. THE RATE OF INACTIVATION BY HEAT OF PEROXIDASE IN MILK. I.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received Nov. 16th, 1914.*)

The presence of peroxidase in fresh cow's milk can be detected by the usual reagents for that enzyme. Hydrogen peroxide and *p*-phenylenediamine are considered the most sensitive reagents for the purpose and are mostly employed in testing milk for peroxidase (Storch's reaction). A dark blue-grey colour is imparted almost instantaneously to the milk if the reaction is positive.

That peroxidase is an actual constituent of the milk, and not due to bacterial contamination, is evident from the fact that initially sterile cow's milk, or milk rendered sterile directly after milking by antiseptics like chloroform or toluene, gives a positive reaction. On the other hand, bacterial cultures from the most common organisms in milk fail to react for peroxidase [Lane-Claypon, 1913, p. 11].

Everything seems to point to the fact that the enzyme is associated with the albumin fraction of the milk. Julius Meyer [1910] showed that when caseinogen is brought down by means of trichloracetic acid, acetic acid, natural acidity or calcium chloride, and properly washed, it does not react for peroxidase, while casein brought down by means of rennet gives but a faint reaction. Again, when milk is centrifuged the enzyme remains in the fluid and does not go up with the cream [Lane-Claypon, 1913, p. 12]. Yet when the precipitate brought down from whey by saturating it with ammonium sulphate is redissolved, the solution reacts very intensely for peroxidase [Lane-Claypon, 1913, p. 10].

Like most enzymes, peroxidase can be inactivated by heat, or at least, when the milk is heated beyond certain temperatures for some time no perceptible reaction is manifested when the usual reagents are employed.

This fact Storch found could be utilised as a reliable test for detecting whether the milk had been heated to 80° C.

Van Eck [1911], investigating the process of inactivation of peroxidase in milk by heat, found that it proceeds as a reaction of the first order and, making use of the formula for a unimolecular reaction, was able to obtain a constant. This was confirmed by the author, who further investigated the matter with the view of ascertaining whether the peroxidase reaction in milk could be employed with any certainty as a test for pasteurisation. The temperature coefficient for the inactivation of peroxidase in milk and the influence of acidity and alkalinity and neutral salts on the rate of inactivation were studied.

Experimental Method.

The technique employed was very similar to that used by van Eck. There is no method for the determination of the absolute quantity of peroxidase, and therefore the amount of the enzyme present in the samples was determined relative to that of the raw milk used in the experiment, and expressed as a percentage of the same. It was carried out colorimetrically. For the standard scale 38 test tubes were used, each containing from 0·1 cc. to 5 cc. of raw milk and made up to 10 cc. with boiled milk. The experimental tubes contained 5 cc. of the sample under investigation, and 5 cc. of boiled milk. To each of the tubes, both experimental and scale, 0·3 cc. of 1 % hydrogen peroxide and 0·25 cc. of 2 % *p*-phenylenediamine were added. Half an hour was allowed to elapse between the addition of the reagent and the comparison of the samples with the scale. After that period no perceptible change could be discerned to take place in the shades of the tubes during the time of the estimation. A fresh scale was made up for each experiment and the raw milk employed invariably was of the same sample as the milk used for investigation. New solutions of hydrogen peroxide and *p*-phenylenediamine were also made up every time, for the former is very unstable and the latter, like guaiacum tincture, after remaining for some time in solution, will react with milk without the addition of hydrogen peroxide. The experimental error of this method varies from 5 % when the peroxidase content is high to 2 % when the amount of peroxidase to be estimated is low.

The milk for investigation was heated in a beaker covered with a cork lid, through which passed a thermometer graduated in tenths of a degree and a stirrer, and which was provided with an opening for the removal of

the samples. The beaker was placed in an asbestos-jacketed water-bath, having a layer of liquid paraffin on the surface to diminish the evaporation, and regulated by a toluene regulator. Like the beaker, the bath contained a stirrer driven by a water-motor. In starting the experiment the milk in the beaker was quickly brought up to the desired temperature in a boiling water-bath, and then without loss of time placed in the regulated bath and the stirrers set going. As soon as the temperature in the beaker became constant the first sample was taken. The taking of the samples was then repeated after the desired intervals. In removing the samples warmed pipettes were used in order not to cool down the milk in the beaker. The test tubes containing the freshly withdrawn samples were immediately placed in cooled water so that the temperature of the milk was rapidly brought down. The entire procedure of removing the samples was carried out regularly in ten seconds.

Determination of the Temperature Coefficient.

In order to determine the temperature coefficient of the rate of inactivation by heat of the peroxidase in milk, it was necessary to ascertain the constants for several temperatures from which the coefficients could be calculated. Preliminary experiments showed that heating of milk at 65° C. for four hours made hardly any perceptible impression on the peroxidase content. On the other hand, at 72° C. the inactivation of the enzyme proceeded at a rate much too fast for any reliable results to be recorded. Temperatures of 71°, 70° and 69° were found to be very convenient and were accordingly chosen for the determination of the constants. The following tables give the results obtained for these three temperatures.

TABLE I.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·005066
0	69·05° C.	63	77
20	68·95	61	61
40	68·9	49	48·3
60	68·9	37	38·2
80	68·9	31	30·3
101	68·9	25	24
120	68·85	19	19
140	68·85	17	15

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}005066$ where $t = 100$ mins., $C_1 = 61$, $C_2 = 19$

TABLE II.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01333
0	70·25° C.	61	55·7
10	70·1	41	41
20	70·1	30	30·1
50	70·07	12·5	12
60	70·05	9·5	8·8
70	70·05	6·5	6·5
80	70·05	5·5	4·8

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}01333$ where $t = 60$ mins., $C_1 = 41$, $C_2 = 6{\cdot}5$.

TABLE III.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·02698
0	71·15° C.	37	35·5
5	71·08	26	26
10	71·05	18	19·1
15	71	13	13·9
20	70·98	9·5	10·2
25	70·98	8	7·5
30	70·9	5·5	5·5
35	70·9	4·5	4

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}02698$ where $t = 25$ mins., $C_1 = 26$, $C_2 = 5{\cdot}5$.

The last column in these tables gives the percentage of peroxidase calculated from the constant (K) obtained from the equation of a unimolecular reaction. The first and last readings were not utilised for calculating the constant, as the possibility of error was more likely in these cases than in any other. In the first sample the temperature is usually not quite constant and the high content of the enzyme makes accurate estimation harder than in the other samples, while in the last sample the experimental error of the colorimetric method is too significant in comparison with the low peroxidase content to be determined. The second reading and the last but one were therefore employed. Considering that the method which was employed for the estimation of the enzyme cannot claim a very high degree of accuracy, the figures calculated from the constants agree well with the figures actually obtained by experiment and this shows clearly that the inactivation of the enzyme proceeds as a reaction of the first order. It is also to be observed that constants obtained by calculating from different data in the tables gave results which approximated to those given above. The error in

the constant is probably between 5–10 %. Fig. 1 gives a graphic representation of the rates of inactivation at the three temperatures.

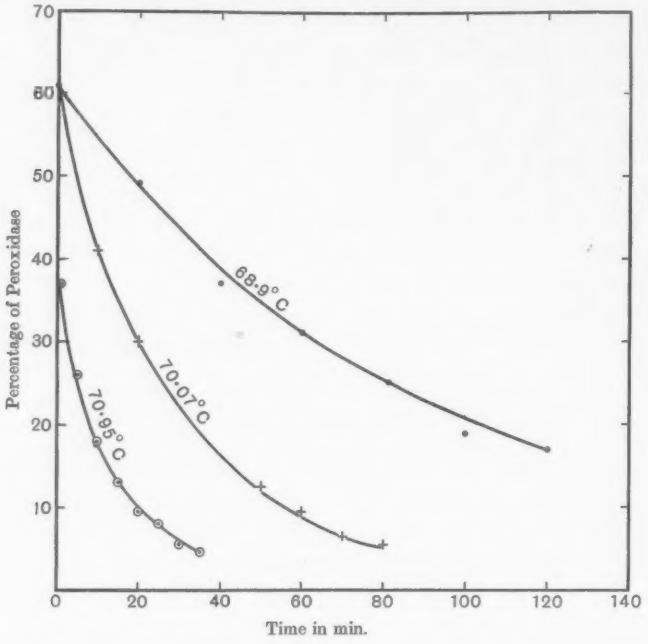


Fig. 1.

Calculating the temperature coefficient from the three constants, we obtain the following figures:

TABLE IV.

Temperature	Constant	Difference in temperature in degrees	Temperature coefficient for one degree
68·9° C.	0·005066	—	—
70·075	0·01333	1·175	2·278
70·95	0·02698	0·875	2·18
70·95	0·02698	2·05	2·236
Mean temperature coefficient			2·231

The mean temperature coefficient is therefore 2·23 per degree. This high value is of the same order as that found by Famulener and Madsen [1908] for the inactivation of tetanolysin and vibriolysin nasik by hot water. For the former the authors obtained the value of 2·4 and for the latter a value

a little under 2 for one degree. Chick and Martin [1910] also found the temperature coefficient for the coagulation of egg albumin by heat to be equal to 1·9 per degree.

The results of Tables I, II and III moreover show the impossibility of applying the peroxidase reaction as a direct test for pasteurisation. The term "pasteurisation" has a very broad application as to the temperature and time of heating the milk. It is not uncommon in some dairies in "pasteurising" to heat the milk up to 80° C. (176° F.), but according to the definition laid down by the Department of Health of the City of New York, pasteurisation consists in heating the milk at not less than

158° F. (70° C.)	for at least	3 mins.
155° F. (68·3° C.)	,,	5 ,,
152° F. (66·7° C.)	,,	10 ,,
148° F. (64·4° C.)	,,	15 ,,
145° F. (62·8° C.)	,,	18 ,,
140° F. (60° C.)	,,	20 ,,

From the constant obtained above for 70° C. it can be calculated that 91·2 % of the original peroxidase would remain after heating the milk for 3 minutes at 158° F. (70° C.). Such an amount of peroxidase could be differentiated with difficulty by the colorimetric method from the full amount of 100 %, even if the original sample of raw milk could be used for the scale. In testing milk, however, for pasteurisation the original raw milk is never available and although different samples of raw milk differ apparently but little in intensity in the peroxidase reaction, the possibility of small variations is by no means excluded. This would make the differentiation still more uncertain. As regards the lower temperature, 20 minutes heating at 60° C. would make no perceptible impression, since, as we have seen, 4 hours at 65° C. makes hardly any difference to the peroxidase content of the milk. It is then evident that the peroxidase reaction cannot be applied at all as a test for pasteurisation. It may, however, serve as an indication whether "pasteurised" milk has been overheated.

TABLE V.

Temperature	Constant	Time in mins. necessary to bring peroxidase down to 10 %	Time in mins. necessary to bring peroxidase down to 50 %
70° C.	0·01333	75	22·5
71	0·02973	33	10·3
72	0·06634	15	4·6
73	0·14802	6·8	2
74	0·33022	3	under a minute
75	0·73673	1·3	,, ,,
80	40·72004	under a minute	,, ,,

Table V gives the periods of time for which the milk must be heated at different temperatures between 70° C. and 80° C. in order to bring down the peroxidase content to 10 % and 50 % respectively. The calculations are made on the assumption that the temperature coefficient remains the same as the temperature rises and the constants in the table are obtained on that assumption. It is clear from the figures that at temperatures beyond 71° C. very short periods of heating produce well marked impressions on the peroxidase content of milk.

Influence of Acidity and Alkalinity on the Rate of Inactivation of Peroxidase in Milk.

Experiments were instituted with the object of studying the effect of acidity and alkalinity on the rate of inactivation of peroxidase in milk. The H ion concentration of the milk was determined by the gas chain method. Unfortunately the determination of H ion concentration in a medium like milk is fraught with many technical difficulties, and the results obtained, though definitely showing the general effect of acidity and alkalinity, are not exact enough for publication in detail. The result arrived at is that a small increase in acidity retards and in alkalinity accelerates the rate of inactivation. Thus the addition of such a quantity of lactic acid as changed the P_H^+ of the milk from about 6·57 to 5·75 brought down the constant at 70° C. from 0·01333 to 0·01086, while the addition of potash which changed the P_H^+ to about 8·26 increased the rate of inactivation at the same temperature to such an extent that the peroxidase content of the milk came down to 2 % in the preliminary heating.

Influence of Salts on the Rate of Inactivation of Peroxidase in Milk.

It was observed by Chick and Martin [1911] that the addition of sodium chloride and ammonium sulphate retarded the rate of coagulation of egg-albumin by heat. It was therefore desirable to ascertain the influence of salts on the rate of inactivation of peroxidase in milk by the same agency. From preliminary experiments it was observed that the addition of sodium chloride greatly retarded the rate of inactivation. In milk made up to the concentration of a N/1 NaCl solution the inactivation of the peroxidase at 70° C. was brought down to a rate too slow for convenient observation, and therefore N/10 and N/20 solutions were used in the actual experiments. The addition of salts alters the tone of the colouration produced by the

peroxidase reaction and salt was therefore added to the scale tubes in the same proportion as to the sample under investigation. Tables VI, VII and VIII illustrate the action of sodium chloride and Fig. 2 shows the same results graphically.

TABLE VI.

(N/10 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·002063
0	69·9° C.	72·5	72·5
30	69·9	59	62·9
90	69·9	48	47·4
120	69·9	42	41·1
150	69·9	36	35·6
180	69·9	32	30·9

TABLE VII.

(N/20 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·003995
0	70·2	72·5	72·5
31	70·15	50	54·5
60	70·1	41	41·8
90	70	31	31·7
120	70	25	24·1
150	70	20	18·25

TABLE VIII.

(*Normal Milk, no salt added.*)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01721
0	69·9	62·5	62·5
5	70·0	50	51·3
10	70·0	40	42
25	70·1	24	23·2
40	70·1	14	12·8
55	70·2	8	7
70	70·2	4	3·9

Calculating $K = \frac{1}{t} \log \frac{C_1}{C_2}$ from different points on the curves in Fig. 2 we get the following mean coefficients for the corresponding concentrations:

N/10 NaCl, K = 0·002063.
N/20 NaCl, K = 0·003995.
Normal Milk, K = 0·01721.

It is seen from the above figures that the concentration of N/20 salt reduces the rate to about one quarter and N/10 to about one eighth. The constant for the normal milk is rather higher than the one usually obtained for a similar temperature, but the same sample of milk was used for the experiments with the N/10 NaCl and N/20 NaCl and, consequently, these results would also be proportionately higher.

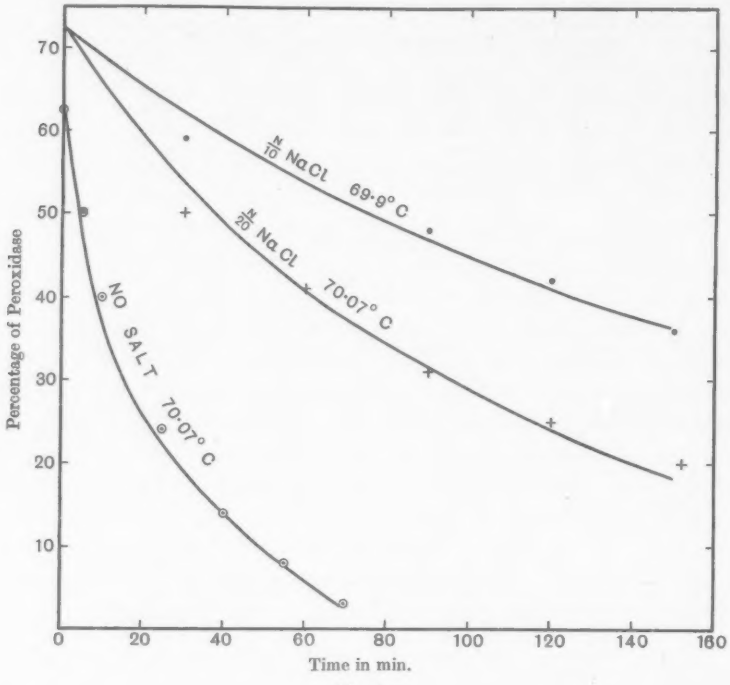


Fig. 2.

The next thing to ascertain was whether other salts had a retarding effect on the rate of inactivation, and if so whether that influence was specific or otherwise. The number of different salts used had to be restricted as some, even in such dilutions as N/20, bring down the caseinogen at 70° C. immediately, or after some time. In all the cases tried the addition of the salts, in concentration of N/20, had a marked retarding effect, although the extent of that influence varied in most cases. The influence of some of the salts is shown by the results in the following tables. Fig. 3 gives curves expressing these results as well as those in Tables VII and VIII.

TABLE IX.

(N/20 KCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00526
0	70·4° C.	65	65
15	70·5	56	54·2
30	70·5	51	45·2
45	70·5	38	37·7
60	70·5	29	31·4
75	70·5	24	26·2
90	70·5	20	21·8

TABLE X.

(N/20 Na_2SO_4.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00546
0	70·35° C.	77·5	75·5
15	70·32	62·5	62·5
30	70·35	53	51·8
45	70·3	42	42·9
60	70·3	33	35·5
75	70·3	28	29·4
90	70·3	20	24·3

TABLE XI.

(N/20 KNO_3.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00981
0	70·2° C.	72·5	82·8
15	70·4	59	59
30	70·35	40	42
45	70·4	28	30
60	70·4	20	21·35
75	70·35	16	15·2
90	70·4	10	10·8

Calculating the constants from the curves of Fig. 3 we get the following figures:

N/20 NaCl K = 0·00399.
N/20 KCl K = 0·00526.
N/20 Na_2SO_4 K = 0·00546.
N/20 KNO_3 K = 0·00981.
Normal milk K = 0·0225.

The temperature for the normal milk and the N/20 NaCl was slightly lower than for the remainder. The constant of the normal milk has been calculated for the right temperature with the help of the temperature coefficient. The temperature coefficient for N/20 NaCl has not been deter-

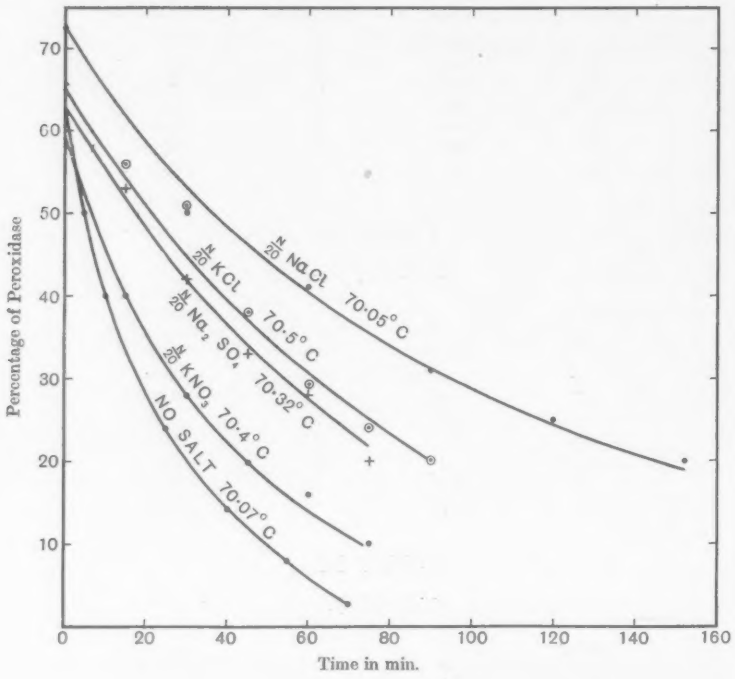


Fig. 3.

mined and consequently no correction could be made for that constant. The results seem to point to the fact that the influence is specific and is not dependent solely on the valency of the ions. KNO_3 and KCl contain monovalent kations and anions, but their constants differ considerably. On the other hand, KCl and Na_2SO_4 contain anions of different valency—the former monovalent and the latter divalent—yet their constants are practically the same.

The Influence of Salts on the rate of Inactivation of Peroxidase in Whey.

The possibility that the salts act on the caseinogen, which then in its altered state influences the rate of inactivation of the peroxidase, was not excluded by the foregoing results and accordingly the effect of the addition of salts to whey was investigated. The whey was prepared by curdling the milk with rennet and filtering it through a cheese-cloth. The experiments were then carried out in the same way as with the milk. The inactivation of the peroxidase in the whey was also observed to proceed as a reaction of the first order but more rapidly than in the milk at the same temperature. The intensity of the peroxidase reaction in the whey was stronger than in the milk, and it was found convenient to dilute it (1 part whey with 3 parts boiled distilled water).

TABLE XII.

(*Diluted Whey, no addition of Salt.*)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01485
0	69·2° C.	72·5	72·5
15	69·35	47	43·4
30	69·3	28	26
45	69·3	14	15·6
60	69·3	9	9·3
75	69·3	5	5·5

TABLE XIII.

(*Diluted Whey and* N/20 NaCl).

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00344
0	69·3° C.	72·5	72·5
15	69·3	62·5	64·3
30	69·3	55	57·2
45	69·3	50	50·8
60	69·3	42	45·1
75	69·3	40	40
90	69·3	38	35·5

Tables XII and XIII and Fig. 4 show the influence of N/20 NaCl on the diluted whey. As with milk, the retarding effect is well marked. N/20 KNO_3 was also found to produce a retarding effect.

Whatever the nature of the action of the salts may be, it is thus seen to be independent of the presence of the caseinogen and fat. Further investigation, which is now in progress, is needed in order to ascertain the nature of that action, and whether the enzyme is influenced directly or indirectly by the presence of the salts.

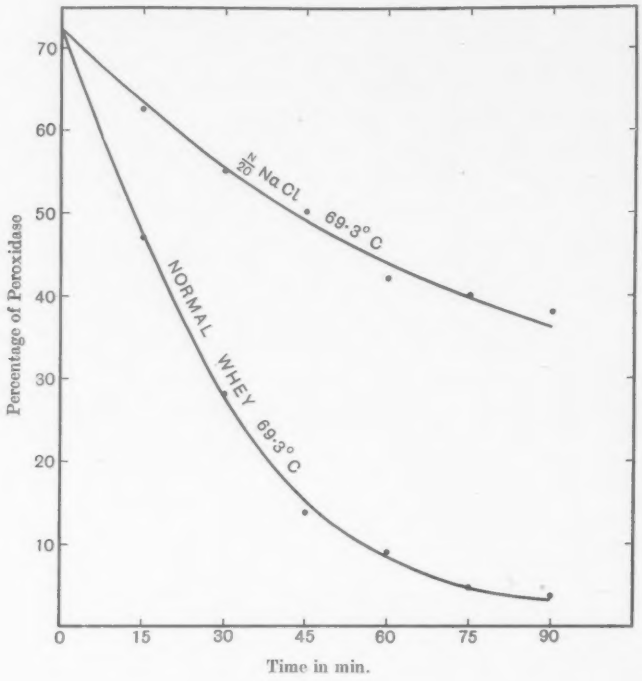


Fig. 4.

Summary.

1. The temperature coefficient for the inactivation of peroxidase in milk by heat is found to be 2·23 per degree centigrade, a value of about the same order as the temperature coefficients for the inactivation by heat of principles like tetanolysin and vibriolysin nasik and for the rate of coagulation of egg-albumin.

2. The rate of inactivation of peroxidase in milk below 70° C. is so small that it does not afford the opportunity of utilising the peroxidase reaction

as a test for pasteurisation. The reaction may, however, indicate whether "pasteurised" milk has been overheated.

3. Small additions of acid retard and of alkali accelerate the rate of inactivation by heat of peroxidase in milk.

4. The presence of salts retards in a pronounced way the inactivation rate. This retardation varies with different salts and is independent of the valencies of their ions.

5. As in milk, the inactivation of peroxidase in whey proceeds as a reaction of the first order.

6. In whey also the presence of salts has a retarding influence on the rate of the enzyme.

In conclusion I wish to express my indebtedness to Professor Harden, F.R.S., for the valuable advice and assistance rendered during this investigation, and to Dr Onodera for helping me with some of the latter part of the experimental work.

REFERENCES.

Chick and Martin (1910). *J. Physiol.*, **40**, 404.
—— (1911). *J. Physiol.*, **43**, 1.
Van Eck (1911). *Zeitsch. Nahr. Genussm.*, **22**, 393.
Famulener and Madsen (1908). *Biochem. Zeitsch.*, **11**, 186.
Lane-Claypon, J. (1913). *Report to Local Gov. Board upon the Biological Properties of Milk, etc.* (where the literature is quoted).
Meyer, J. (1910). *Arbeit. kaiserl. Gesundheitsamt*, **34**, 115.

LXVI. LIVER NITROGEN IN ANAPHYLAXIS.

By GEORGE BARGER and HENRY HALLETT DALE.

(*Received Nov. 17th, 1914.*)

It has been clearly demonstrated by the work of Manwaring [1910] and of Voegtlin and Bernheim [1911], that events occurring in the liver have, at least in some species, a predominant importance in producing the train of symptoms known as "anaphylactic shock." The nature of these events, and of the changes in the liver rendering it thus specifically sensitive to a previously injected antigen, has hitherto been obscure. That the events are of such a kind as to produce a hindrance to the portal circulation, has been suggested; and there is a probability that such mechanical factors play at least some part. The recent work of Hashimoto and Pick [1914], however, seemed rich in promise of explanation along other lines. According to these observers, the liver of an animal, rendered anaphylactic by a first small injection of a foreign protein, contains a largely increased store of uncoagulable nitrogenous substances, without change of its total nitrogen from the normal average. The result is an increase of the uncoagulable fraction of the total nitrogen from a normal average of about 8 % to 22 %, or even more. According to their finding, the increase becomes definite two or three days after the sensitising injection, and attains the above-mentioned maximum at about the 14th day, thereafter very gradually declining to a lower, but still supernormal ratio, which they found still maintained as late as the 68th day. They further found that the spleen played an important part, in producing and maintaining this excess of uncoagulable nitrogen in the liver of the anaphylactic animal. Sensitisation performed the day after splenectomy, or followed by splenectomy, was not accompanied by this rise in the uncoagulable nitrogen ratio. Hashimoto and Pick's experiments were made on guinea-pigs, and they point out that this accumulation of non-coagulable nitrogen in the liver has no bearing on the plain muscle sensitisation, which gives to the anaphylactic shock in this species its most characteristic features. Their results, however, if confirmed, have an interesting relation to the

anaphylactic shock seen in such animals as the dog, in which the exclusion of the liver from circulation renders the re-injection of the sensitising antigen without effect.

Hashimoto and Pick's observations were especially suggestive in that they seemed to harmonise with other observations on anaphylaxis and similar conditions. Thus it has been shown, by several workers, that the symptoms of the anaphylactic shock correspond closely with those following the injection of certain products of the partial cleavage of proteins. It has been shown also, that a second parenteral administration of a foreign protein is followed by an increased output of nitrogen in the urine, considerably greater than could be accounted for by the nitrogen-content of the injected substance. If the result of a first injection then, were, as stated, to cause an accumulation of protein cleavage-products in the liver cells, and if these were released into the circulation as the result of the second injection, a possibility would be presented of co-ordinating a number of hitherto imperfectly connected observations. With a view to examining these possibilities further, our ultimate object being a closer analysis of the process of sensitisation and anaphylactic shock in the dog, we have repeated Hashimoto and Pick's experiments; but we find ourselves held up, at the outset of the investigation, by a complete failure to confirm their observations. We are at a loss to account for the discrepancy. Our technique has differed from theirs in no essential particular. We have examined in all 13 guinea-pigs sensitised with injections of horse-serum, varying from 0·1 to 2 cc., and have not found one in which the ratio of non-coagulable to total liver-nitrogen lies outside the range of variation seen in normal controls. The experiments on controls were interspersed among the anaphylactic series, so that seasonal or dietary variation would affect both series alike. The average values in the two series are practically identical.

Being unable to confirm the fundamental observation of the presence of excess of uncoagulable nitrogen in the anaphylactic liver, we were not in a position to study the effect, on the alleged excess, of subjecting the animal to anaphylactic shock. Nor have we, save for casual observations of the rate of autolysis for a few hours at room temperature, which showed no significant difference between normal and anaphylactic liver, made any attempt to follow the further observations of Hashimoto and Pick, since these postulate the accuracy of the main observation, which we fail to confirm. Nor have we, as we had proposed, extended the observations to other species.

The publication of results, which are a mere contradiction of those

obtained by previous investigators, without the possibility or near prospect of the personal communication, which we should have desired, and which might have helped us to an explanation of the discrepancy, is at present an unfortunate necessity.

Experimental.

The observations were made on young, healthy guinea-pigs, varying in weight from 210 to 385 g., taken from a common stock, kept under identical conditions. All the observations were made between August 31st and October 14th and the diet (hay, oats, bran and green stuff) was not varied in composition or quantity throughout the series. Nevertheless, to exclude the influence of seasonal variation, or of any unnoticed change in diet, the observations on normal controls were interspersed among the series on anaphylactic animals.

The anaphylactic series consisted of 13 guinea-pigs, each sensitised by a subcutaneous injection of normal horse-serum; five receiving 0·1 cc., six receiving 0·5 cc., and two receiving 2 cc. each. The livers were examined at an interval varying from 11 to 21 days after the injection. One of the six animals sensitised by 0·5 cc. of horse-serum was killed on the 12th day by a second, intravenous injection of 0·5 cc. of the same serum; death took place within five minutes from typical acute shock, with extreme and permanent distension of the lungs. The others were killed by a blow on the head, and in all cases the animal was bled as completely as possible by severing the blood-vessels in the neck and compressing the abdomen. The 12 normal controls were killed in the same manner.

The pale and nearly bloodless liver was removed rapidly after killing and was immediately cut into portions of suitable size for the different determinations. In a few trial experiments on normal livers we used a method identical in all details with that employed by Hashimoto and Pick; the whole liver, after removing the gall-bladder and large vessels, was pounded in a mortar and then rubbed through fine nickel gauze, portions for estimation being weighed out from the semi-fluid pulp so produced. Such a method provided an ideal safeguard against local variations of composition. At the same time it is troublesome and wasteful—the loss in the meshes of the gauze being such as to make it difficult to obtain four adequately large samples from the liver of a small guinea-pig. Drying is also apt to occur in the thinnish film of material produced. We made a few experiments, therefore, to discover whether local variations of composition occur in the

liver of sufficient magnitude to weaken the accuracy of estimates carried out on different samples cut from a whole liver. We found that the values, whether for total or non-coagulable nitrogen, obtained from different pieces of the same liver, were remarkably concordant—showing at least as good a correspondence as those obtained from different portions of the disintegrated and mixed liver substance. The small error was clearly the error of the Kjeldahl method, and not that of imperfect sampling. We, therefore, abandoned the preliminary disintegration, and simply weighed out in clean, tared beakers as many pieces of whole liver substance as were required, merely avoiding the central portions, where large vessels and ducts are present in undue proportion to the liver cells.

For determining total nitrogen pieces of from 0·5 to 1 g. were usually taken, and these were at once washed from the beakers into Kjeldahl flasks. To each flask were added 20 cc. of strong sulphuric acid, 10 g. of potassium sulphate, and a small fragment of copper gauze. A preliminary heating on a water-bath, before burning, was found useful in diminishing the trouble of bumping.

For determinations of non-coagulable nitrogen portions of 2 to 2·5 g. were usually employed. Two different methods of coagulation were used. One of these gave, with some uniformity, slightly lower values than the other. Estimates on both normal and anaphylactic livers were made by both methods, and comparisons were made of normal and anaphylactic values obtained by the same method.

Method I. This is identical with the method employed by Hashimoto and Pick, except in that disintegration is performed after, instead of before weighing. The sample of liver is transferred with a little distilled water to a glass mortar and rubbed up into as fine a pulp as possible. More distilled water is added and rubbed up with the liver-pulp. After a brief settling the supernatant fluid is poured back into the beaker, the residue of less thoroughly ground fragments subjected to renewed rubbing, more water added and poured off; the process is repeated till nothing remains but a few fine colourless shreds. These are finally washed into the beaker and the mortar and pestle thoroughly washed with several more small quantities of distilled water, which are added to the mixture. The fine emulsion thus obtained was made up, as in Hashimoto and Pick's experiments, to about 70 cc. A pinch of salt was added and the beaker transferred to a boiling water-bath; three drops of 20 % acetic acid were then added from a fine pipette, and heating continued till coagulation was quite complete.

TABLE I.

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse-serum and time since injection	Number of liver-sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)	Method for non-coagulable nitrogen	Milligrams of nitrogen	Percentage of nitrogen	Mean	Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total nitrogen by Method I, after standing 3 hours
1 Aug. 31st	320	None (control)	1	1·174	Total		35·07	2·99	2·97			
			2	1·680	,,		49·35	2·94				
			3	1·047	Non-coagulable	I	3·36	0·321	0·298	10		
			4	1·630	,,	,,	4·48	0·275				
2 Sept. 1st	2 guinea-pigs, each about 250 grammes. Livers mixed	None (control)	1	2·017	Total		62·37	3·07	3·12			
			2	1·753	,,		55·65	3·17				
			3	2·709	Non-coagulable	I	7·65	0·282	0·286	9·2		
			4	3·025	,,	,,	8·78	0·290				
3 Sept. 3rd	280	None (control)	1	1·593	Total		53·55	3·36	3·36			
			2	1·738	Non-coagulable	II	4·57	0·263	0·260		7·7	
			3	1·880	,,	,,	4·85	0·258				
4 Sept. 8th	390	None (control)	1	3·211	Total		94·92	2·96	2·96			
			2	2·084	,,		61·74	2·96				
			3	1·891	Non-coagulable	II	4·2	0·222	0·222		7·5	
			4	1·790	Lost by accident							
5 Sept. 9th	305	None (control)	1	1·363	Total		48·8	3·58	3·56			
			2	1·497	,,		53·0	3·54				
			3	1·203	Non-coagulable	II	3·7	0·30	0·30		8·4	
			4	1·522	,,	,,	4·6	0·30				
6 Sept. 11th	220	0·1 cc. 11 days	1	1·766	Total		62·37	3·53	3·48			
			2	0·2638	,, (Micro Kjeldahl)		9·1	3·44			7·9	
			3	1·800	Non-coagulable	II	4·95	0·275	0·274			
			4	1·792	,,	,,	4·90	0·273				
7 Sept. 14th	245	0·1 cc. 14 days	1	0·599	Total		19·32	3·22	3·16			
			2	0·939	,,		29·09	3·10				
			3	1·685	Non-coagulable	II	4·39	0·260	0·251		7·95	
			4	1·945	,,	,,	4·71	0·242				
8 Sept. 16th	209	None (control)	1	0·599	Total		19·22	3·21	3·21			
			2	0·760	,,		24·36	3·21				
			3	2·567	Non-coagulable	I	6·93	0·270	0·267	8·3		
			4	2·570	,,	,,	6·82	0·265				
9 Sept. 16th	245	0·1 cc. 16 days	1	1·070	Total		24·68	2·31	2·32			
			2	0·846	,,		19·64	2·32				
			3	2·632	Non-coagulable	I						

9 Sept. 16th	245	0·1 cc. 16 days	1 2 3 4	1·070 0·846 2·632 2·933	Total ,, Non-coagulable ,,	 I ,,	24·68 19·64 5·78 6·40	2·31 2·32 0·219 0·219	2·32 0·219	9·5		
10 Sept. 17th	280	None (control)	1 2 3 4	0·941 0·826 2·012 2·207	Total ,, Non-coagulable ,,	 I ,,	23·5 20·5 4·4 4·7	2·50 2·48 0·219 0·213	2·49 0·216	8·7		
11 Sept. 17th	251	0·1 cc. 17 days	1 2 3 4	0·928 0·657 2·849 2·347	Total ,, Non-coagulable ,,	 I ,,	31·7 23·3 7·1 6·2	3·42 3·55 0·250 0·265	3·48 0·258	7·4		
12 Sept. 21st	280	None (control)	1 2 3 4	0·873 0·983 2·379 2·459	Total ,, Non-coagulable ,,	 I ,,	27·8 30·7 5·65 5·8	3·18 3·12 0·238 0·236	3·15 0·237	7·5		
13 Sept. 21st	270	0·1 cc. 21 days	1 2 3 4	1·112 1·172 2·327 2·543	Total ,, Non-coagulable ,,	 I ,,	40·0 43·0 6·5 6·8	3·60 3·67 0·279 0·267	3·63 0·273	7·5		
14 Sept. 29th	310	0·5 cc. 11 days	1 2 3 4	1·490 1·623 3·054 2·151	Total ,, Non-coagulable ,,	 I ,,	45·0 50·4 8·0 5·8	3·02 3·10 0·264 0·270	3·06 0·267	8·7		
15 Sept. 30th	290	0·5 cc. 12 days	1 2 3 4	1·548 1·265 2·441 2·247	Total ,, Non-coagulable ,,	 I ,,	48·9 41·4 6·15 5·75	3·16 3·27 0·252 0·256	3·22 0·254	7·9		
16 Oct. 1st	294	0·5 cc. 13 days	1 2 3 4	1·055 1·609 2·255 2·613	Total ,, Non-coagulable ,,	 I ,,	41·3 61·6 6·2 7·2	3·90 3·82 0·275 0·275	3·86 0·275	7·1		
17 Oct. 1st	278	None (control)	1 2 3 4	0·849 0·980 2·401 2·494	Total ,, Non-coagulable ,,	 I ,,	30·8 34·9 5·95 6·00	3·62 3·56 0·247 0·241	3·59 0·244	6·8		
18 Oct. 2nd	385	0·5 cc. 14 days	1 2 3 4 5 6	0·978 0·951 2·871 2·464 2·077	Total ,, Non-coagulable ,, ,, Lost by accident	 I ,, II	23·2 22·7 6·8 5·6 4·5	2·37 2·38 0·250 0·227 0·217	2·38 0·238 0·217	10	9·1	
19 Oct. 5th	285	0·5 cc. 17 days	1 2 3 4 5 6	0·534 0·411 2·040 2·133 1·895 1·735	Total ,, Non-coagulable ,, ,, Lost by accident	 II ,, I ,,	18·3 13·5 5·1 5·2 5·0	3·43 3·29 0·250 0·244 0·264	3·36 0·247 0·264	7·9	7·3	

TABLE I—*continued.*

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse-serum and time since injection	Number of liver sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)	Method for non-coagulable nitrogen	Milligrams of nitrogen	Percentage of nitrogen	Mean	Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total nitrogen by Method I, after standing 3 hours
20 Oct. 7th	310	0·5 cc. 19 days	1	0·747	Total		17·92	2·40	2·38	9·4	9·1	12·2
			2	0·704	,,		16·66	2·37				
			3	2·318	Non-coagulable	I	5·25	0·226	0·225			
			4	2·408	,,	,,	5·4	0·224				
			5	2·327	,,	II	5·0	0·215	0·216			
			6	2·205	,,	,,	4·8	0·218				
			7	2·004	,,	I	5·7	0·284	0·291			
			8	2·009	,, (After 3½ hrs. autolysis)	,,	6·0	0·298				
21 Oct. 8th	275	2 cc. 15 days	1	0·440	Total		16·2	3·70	3·65	8·2	7·5	
			2	0·610	,,		22·0	3·61				
			3	2·244	Non-coagulable	I	6·8	0·303	0·299			
			4	2·242	,,	,,	6·6	0·294				
			5	1·274	,,	II	3·6	0·283	0·275			
			6	1·633	,,	,,	4·35	0·266				
22 Oct. 8th	265	2 cc. 15 days	1	0·593	Total		21·4	3·61	3·59	10·1	8·7	
			2	0·909	,,		32·3	3·57				
			3	1·751	Non-coagulable	I	6·35	0·363	0·363			
			4	2·231	,,	,,	8·1	0·363				
			5	1·659	,,	II	5·3	0·319	0·313			
			6	1·793	,,	,,	5·5	0·307				
23 Oct. 9th	360	None (control)	1	0·594	Total		21·80	3·67	3·65	8·3	7·9	
			2	0·726	,,		26·32	3·63				
			3	1·738	Non-coagulable	I	5·4	0·311	0·302			
			4	1·915	,,	,,	5·6	0·293				
			5	2·158	,,	II	6·2	0·287	0·290			
			6	2·219	,,	,,	6·5	0·293				
24 Oct. 12th	390	None (control)	1	1·135	Total		29·43	2·60	2·64	9·2	8·3	11·2
			2	0·577	,,		15·47	2·68				
			3	2·584	Non-coagulable	II	5·63	0·218	0·218			
			4	2·638	,,	,,	5·75	0·218				
			5	2·505	,,	I	6·2	0·247	0·243			
			6	2·495	,,	,,	5·95	0·239				
			7	2·612	,,	,,	8·4	0·283	0·295			
			8	2·374	,, (After 3 hrs. autolysis)	,,	8·2	0·307				
25 Oct. 13th	260	None (control)	1	0·785	Total		25·97	3·31	3·27	11·3		
			2	1·155	,,		37·31	3·22				
			3	2·105	Non-coagulable	I	8·2	0·389	0·369			
			4	2·137	,,	,,	7·45	0·349				

The time on the water-bath was in all cases about 30 minutes. The beaker and its contents were then cooled, the coagulum carefully broken up with a rod into fine fragments, and the whole, with repeated washings, transferred to a 100 cc. standard flask, the volume being then accurately adjusted with distilled water. The flask was well shaken and the contents filtered through an ordinary folded filter paper.

Method II. The piece of liver, as soon as it was weighed, was partially coagulated whole, by pouring into the beaker about 10 cc. of boiling distilled water faintly acidulated with acetic acid, heating on the water-bath for one minute and then cooling. By this preliminary coagulation the disintegration in the mortar was rendered much easier. The piece of liver was ground first with a small quantity, and then with the whole of the fluid in which it was coagulated. A fine creamy emulsion was obtained, and, by pouring off after brief settling and regrinding the residue with successive portions of distilled water, a suspension of the whole substance in a very fine state of division was obtained. The fluid was again made up to 70 cc. and definitive coagulation carried out on the same lines as in Method I. The transference of the fluid and coagulum to the standard flask was rendered easier in this case by the finely divided state of the coagulum. The volume was made up to 100 cc. and filtration carried out as in Method I.

Of the filtrate obtained by either method a convenient volume (usually 70 cc.) was taken for Kjeldahl estimation. Hashimoto and Pick stipulate that the filtrate shall be free from any trace of opalescence, though they admit, in a footnote, that opalescent filtrates do not differ materially in nitrogen-content from perfectly clear ones. They suggest that the opalescence is due to glycogen, and their suggestion is certainly correct. It may be said, indeed, that with the majority of livers, worked up with sufficient rapidity, a filtrate free from traces of opalescence is not obtainable. This is especially the case with Method II, in which the preliminary scalding effectively stops post-mortem glycogenolysis. The nitrogen value obtained by Method II is, however, regularly somewhat lower than that obtained from the same liver by Method I. It is clear, then, that this opalescence has no connection with imperfect coagulation, and we have ignored it. Glycogen-rich livers, giving opalescent filtrates, naturally show lower percentages of both total and non-coagulable nitrogen than livers which are practically nitrogen-free; but the ratio of non-coagulable to total nitrogen is not disturbed. In every experiment duplicate samples were worked up.

In several cases the non-coagulable nitrogen was estimated by both Methods I and II—two samples being worked up by each method.

The ammonia resulting from the Kjeldahl combustion was distilled into standard sulphuric acid, 10 cc. N/2 acid being used for a total, 10 cc. N/10 acid for a non-coagulable nitrogen determination. Titration was carried out with N/20 caustic potash, methyl red being used as indicator. The standards were carefully checked on several occasions during the series of experiments. The results are given in Table I.

It will be seen that Method I for determination of the non-coagulable nitrogen regularly gives a somewhat higher percentage than Method II, when both are applied to the same liver. For purposes of comparison, therefore, it is more accurate to consider separately the results obtained by the two methods, as in Table II.

TABLE II.

Percentage of Non-coagulable in Total Nitrogen.

Method I.			Method II.		
Normal	Anaphylactic	Sensitising dose and interval	Normal	Anaphylactic	Sensitising dose and interval
10·0	9·5	0·1 cc. 16 days	7·7	7·9	0·1 cc. 11 days
9·2	7·4	0·1 cc. 17 „	7·5	7·95	0·1 cc. 14 „
8·3	7·5	0·1 cc. 21 „	8·4	—	—
8·7	8·7	0·5 cc. 11 „	8·3	—	—
7·5	7·9	0·5 cc. 12 „	9·2	—	—
6·8	7·1	0·5 cc. 13 „	—	—	—
8·3	10·0	0·5 cc. 14 „	—	9·1	0·5 cc. 14 „
9·2	7·9	0·5 cc. 17 „	—	7·3	0·5 cc. 17 „
11·3	9·4	0·5 cc. 19 „	—	9·1	0·5 cc. 19 „
—	8·2	2 cc. 15 „	—	7·5	2 cc. 15 „
—	10·1	2 cc. 15 „	—	8·7	2 cc. 15 „
Average 8·8	8·5	—	8·2	8·2	—

It is sufficiently clear that, whichever method is used, there is no significant difference between the percentages obtained. We are entirely unable to explain the failure of our animals to show the striking difference exhibited by those of Hashimoto and Pick. That our guinea-pigs were satisfactorily sensitised is clear from the rapid death, in response to a second injection, of the one animal thus tested. Being unable to obtain the accumulation of non-coagulable nitrogenous substances which Hashimoto and Pick described, we are not in a position to investigate it further. Our results seem to us, however, sufficiently clear to indicate that, whatever its origin, the condition described by them had no relation to anaphylaxis.

These experiments have been carried out on behalf of the Medical Research Committee, National Health Insurance. The work was done at the Lister Institute of Preventive Medicine, to the Governors and Director of which our warmest thanks are due for their kind hospitality.

REFERENCES.

Hashimoto and Pick (1914). *Zentralbl. Physiol.*, **27**, No. 16; *Arch. exp. Path. Pharm.*, **76**, 89; *Zeitsch. Immunitätsforschung*, **21**, 237.

Manwaring (1910). *Zeitsch. Immunitätsforschung*, **8**, 1.

Voegtlin and Bernheim (1911). *J. Pharm. exp. Therap.*, **2**, 507.

INDEX

Abrus precatorius, colouring matter of seed-coats of (Sarkar) 281
Aceto-acetic acid, excretion of, in aceto-nuria (Kennaway) 355
Acetone-soluble phosphatides, the so-called (MacLean) 453
Acetonuria, relative amounts of β-hydroxy-butyric acid and aceto-acetic acid excreted in (Kennaway) 355
Acetylcholine (Ewins) 44
Acids, fatty, with branched chains, mode of oxidation of (Raper) 320
ACKROYD, H. On the purine metabolism of rats 434
Agglutination, of bacteria by specific sera, mechanism of (Tulloch) 293
Allantoin, estimation of, in urine in presence of glucose (Plimmer and Skelton) 641
Allantoin, indirect estimation of, in urine by urease (Plimmer and Skelton) 70
Amino-acids, Herzig and Meyer's reaction applied to (Burn) 154
Anaphylaxis, liver nitrogen in (Barger and Dale) 670
ANNETT, H. E. The urease content of certain Indian seeds 449
Antirrhinum majus, flower pigments of (Wheldale and Bassett) 204
Aspartic acid, estimation of, in products of protein hydrolysis (Foreman) 463
ATKIN, E. E. The chemical nature of a bacterial haemolysin 328
Auxetic action, of coal tar bases and its inactivation (Norris) 253

Bacteria, mechanism of agglutination of, by specific sera (Tulloch) 293
BARGER, G. and DALE, H. H. Liver nitrogen in anaphylaxis 670
Bases, of coal-tar, auxetic action of (Norris) 253
BASSETT, H. Ll. *see* WHELDALE, M. 204
β-Hydroxybutyric acid, estimation of (Kenna-way) 230
β-Hydroxybutyric acid, excretion of, in acetonuria (Kennaway) 355
Blood, estimation of sugar in (Gardner and MacLean) 391
Blood, remarks on Symons' note on Teich-mann's test for (Sutherland and Mitra) 128
BRAHMACHARI, U. N. An investigation into the physico-chemical mechanism of haemo-lysis by specific haemolysins. No. II. The electrical conductivity of sensitised corpuscles and the action of inorganic ferments or metal-sols upon them 227
Brain, content of cholesterol (Rosenheim) 82
Brain, presence of oxycholesterol and its esters in the (Rosenheim) 74
Brain, the galactosides of (Rosenheim) 110, 121
BURN, J. H. Herzig and Meyer's reaction applied to proteins and amino-acids 154

Casein (Geake) 30
Casein, production of, from caseinogen (Schry-ver) 152
Caseinogen, action of coagulating enzymes on (Harden and Macallum) 90
Caseinogen, action of diazomethane on (Geake and Nierenstein) 287
Caseinogen, formation of a peptone from, by the action of dilute acid in the cold (Funk and McLeod) 107
Caseinogen (Geake) 30
Catalase, of washed zymin and dried yeast (Harden and Zilva) 217
CHICK, H. The apparent formation of euglo-bulin from pseudoglobulin and a suggestion as to the relationship between these two proteins in serum 404
CHICK, H. The viscosity of protein solutions. II. Pseudoglobulin and euglobulin (Horse) 261
CHICK, H. and LUBRZYNSKA, E. Viscosity of some protein solutions 59
Cholesterol, amount present in human and animal brain (Rosenheim) 82
Cholesterol, of the brain (Rosenheim) 74, 82
Choline, some new physiologically active derivatives of (Ewins) 366
Coagulating enzymes, action of, on caseinogen (Harden and Macallum) 90
Coal-tar, auxetic action of bases of (Norris) 253
COLE, S. W. The estimation of lactose and glucose by the copper-iodide method 134
Conductivity, of milk (Jackson and Rothera) 1
COOPER, E. A. The curative action of autolysed yeast against avian poly-neuritis 250
COOPER, E. A. The relations of vitamine to lipoids 347
Corpuscles, sensitised, action of metal-sols upon (Brahmachari) 227
Counter diffusion, in aqueous solution (Osborne and Jackson) 246

CREIGHTON, H. J. M. *see* HARRIS, D. F. 585
CUNNINGHAM, M. and DORÉE, C. The production of ω-hydroxy-s-methylfurfuraldehyde from carbohydrates and its influence on the estimation of pentosans and methylpentosans 438

DALE, H. H. *see* BARGER, G. 670
Diastatic enzymes, hydrolysis of glycogen by (Norris) 421
Diazomethane, action of, on caseinogen (Geake and Nierenstein) 287
DORÉE, C. *see* CUNNINGHAM, M. 438
DRUMMOND, J. C. and FUNK, C. The chemical investigation of the phosphotungstate precipitate from rice-polishings 598
DRUMMOND, J. C. *see* ROSENHEIM, O. 143
DRUMMOND, J. M. A contribution to the study of a proteolytic organism 38

EDIE, E. S. Action of pepsin and trypsin on one another 193
EDIE, E. S. On the resistance of trypsin solutions to heat 84
Enzymes, coagulating, action on caseinogen (Harden and Macallum) 90
Enzymes, diastatic, hydrolysis of glycogen by (Norris) 421
Enzymes of washed zymin and dried yeast (Harden and Norris) 100
Enzymes of washed zymin and dried yeast (Harden and Zilva) 217
Ergot, occurrence of acetylcholine in (Ewins) 44
Esters, of oxycholesterol, presence of, in the brain (Rosenheim) 74
Euglobulin, relationship of, to pseudoglobulin in serum (Chick) 404
Euglobulin, viscosity of solutions of (Chick) 261
EWINS, A. J. Acetylcholine, a new active principle of ergot 44
EWINS, A. J. Some new physiologically active derivatives of choline 366
EWINS, A. J. The constitution of pseudomuscarine ("synthetic muscarine") 209

Fish, fresh-water, respiratory exchange in (Gardner and Leetham) 374, 591
Flower pigments of *Antirrhinum majus* (Wheldale and Bassett) 204
FOREMAN, F. W. The transformation of glutaminic acid into *l*-pyrrolidonecarboxylic acid in aqueous solution 481
FOREMAN, F. W. Quantitative estimation of aspartic and glutaminic acids in the products of protein hydrolysis 463
Freezing point, of milk (Jackson and Rothera) 1
FUNK, C. and MCLEOD, J. W. The formation of a peptone from caseinogen by the prolonged action of dilute hydrochloric acid in the cold 107
FUNK, C. *see* DRUMMOND, J. C. 598

Galactosides of the brain (Rosenheim) 110, 121
GARDNER, A. D. and MACLEAN, H. A method for the estimation of sugar in blood with observations on some modern methods 391
GARDNER, J. A. and LEETHAM, C. On the respiratory exchange in fresh-water fish. Part I. On brown trout 374
GARDNER, J. A. and LEETHAM, C. On the respiratory exchange in fresh-water fish. Part II. On brown trout 591
GEAKE, A. and NIERENSTEIN, M. The action of diazomethane on caseinogen 287
GEAKE, A. Caseinogen and casein 30
Gelatinisation of pectin (Haynes) 553
Glucose, estimation of, by the copper-iodide method (Cole) 134
Glutaminic acid, estimation of, in products of protein hydrolysis (Foreman) 463
Glutaminic acid, transformation of, into *l*-pyrrolidonecarboxylic acid in aqueous solution (Foreman) 481
Glycogen, factors influencing the end-point of the hydrolysis of, by diastatic enzymes (Norris) 421

Haemolysin, chemical nature of a bacterial (Atkin) 328
Haemolysis, mechanism of (Brahmachari) 227
HARDEN, A. and MACALLUM, A. B. On the action of coagulating enzymes on caseinogen 90
HARDEN, A. and NORRIS, R. V. The enzymes of washed zymin and dried yeast (Lebedeff). II. Reductase 100
HARDEN, A. and ZILVA, S. S. The enzymes of washed zymin and dried yeast (Lebedeff). III. Peroxidase, catalase, invertase and maltase 217
HARRIS, D. F. and CREIGHTON, H. J. M. The action of poisons on reductase and attempts to isolate this enzyme 585
HARTLEY, P. The determination of the composition of the different proteins of ox and horse serum by the method of van Slyke 541
HAYNES, D. The gelatinisation of pectin in solutions of the alkalies and the alkaline earths 553
Heat, rate of inactivation of peroxidase in milk by (Zilva) 656
Heat, resistance of trypsin solutions to (Edie) 84
Herzig and Meyer's reaction, applied to proteins and amino-acids (Burn) 154
Hydrogen electrode, an improved (Walpole) 131
Hydrolysis of proteins (Pittom) 157
ω-Hydroxy-s-methylfurfuraldehyde, production of, from carbohydrates and its influence on the estimation of pentosans and methylpentosans (Cunningham and Dorée) 438

Indicators, recent, notes on (Walpole) 628
Invertase of washed zymin and dried yeast (Harden and Zilva) 217

JACKSON, L. C. and ROTHERA, A. C. H. Milk —its milk sugar, conductivity and depression of freezing point 1
JACKSON, L. C. *see* OSBORNE, W. A. 246

KENNAWAY, E. L. On the estimation of β-hydroxybutyric acid 230
KENNAWAY, E. L. The relative amounts of β-hydroxybutyric acid and aceto-acetic acid excreted in acetonuria 355
Kerasin, preparation of, from brain (Rosenheim) 110
KINCAID, H. E. *see* OSBORNE, W. A. 28

Lactose, estimation of, by the copper-iodide method (Cole) 134
LEETHAM, C. *see* GARDNER, J. A. 374, 591
Lipoids, relations of vitamine to (Cooper) 347
Liquid crystals (Rosenheim) 121
l-Pyrrolidonecarboxylic acid, formation of, from glutaminic acid in aqueous solution (Foreman) 481
LUBRZYNSKA, E. *see* CHICK, H. 59

MACALLUM, A. B. *see* HARDEN, A. 90
MACLEAN, H. *see* GARDNER, A. D. 391
MACLEAN, H. On the so-called "acetone-soluble phosphatides" 453
MCLEOD, J. W. *see* FUNK, C. 107
Maltase of washed zymin and dried yeast (Harden and Zilva) 217
Milk, milk-sugar, conductivity and freezing point of (Jackson and Rothera) 1
Milk, rate of inactivation by heat of peroxidase in (Zilva) 656
Milk sugar, of milk (Jackson and Rothera) 1
MITRA, G. C. *see* SUTHERLAND, W. D. 128
Muscarine, "synthetic," constitution of (Ewins) 209

NIERENSTEIN, M. *see* GEAKE, A. 287
NORRIS, D. A note on the bases of gasworks coal-tar which are believed to be the predisposing cause of pitch cancer, with special reference to their action on lymphocytes, together with a method for their inactivation. Part I. Auxetic action 253
NORRIS, R. V. The hydrolysis of glycogen by diastatic enzymes. Part III. Factors influencing the end-point of the hydrolysis 421
NORRIS, R. V. *see* HARDEN, A. 100

ORR, J. B. The influence of excessive water ingestion on protein metabolism 530
OSBORNE, W. A. and JACKSON, L. C. Counter diffusion in aqueous solution 246
OSBORNE, W. A. and KINCAID, H. E. Osmotic phenomena of yolk of egg 28
Osmotic phenomena of yolk of egg (Osborne and Kincaid) 28
Oxidation, mode of, of certain fatty acids with branched chains (Raper) 320
Oxycholesterol, presence of, and its esters in the brain (Rosenheim) 74

PEARSON, A. L. A comparison between the molecular weights of protagon and of the phosphatide and cerebrosides obtainable from it 616
Pectin, gelatinisation of, in solutions of the alkalies and alkaline earths (Haynes) 553
Pepsin, action on trypsin (Edie) 193
Peptone, formation of, from caseinogen by the action of dilute acid in the cold (Funk and McLeod) 107
Peroxidase of washed zymin and dried yeast (Harden and Zilva) 217
Peroxidase, rate of inactivation by heat of, in milk (Zilva) 656
Phosphatides, the so-called "acetone-soluble" (MacLean) 453
Phosphorus, gravimetric estimation of minute quantities of (Raper) 649
Phrenosin, melting point of (Rosenheim) 121
Phrenosin, preparation of, from brain (Rosenheim) 110
Pigment, the black, in the skin of an Australian black (Young) 460
PITTOM, W. W. P. Studies in protein hydrolysis 157
PLIMMER, R. H. A. and SKELTON, R. F. The estimation of allantoin in urine in the presence of glucose 641
PLIMMER, R. H. A. and SKELTON, R. F. The quantitative estimation of urea and indirectly of allantoin in urine by means of urease 70
Poisons, action of, on reductase (Harris and Creighton) 585
Polyneuritis, avian, curative action of autolysed yeast against (Cooper) 250
PORTER, A. E. On the behaviour of trypsin in the presence of a specific precipitate 50
Protagon, molecular weight of, compared with those of phosphatide and cerebrosides obtainable from it (Pearson) 616
Protein, estimation of aspartic and glutaminic acids in products of hydrolysis of (Foreman) 463
Protein, hydrolysis (Pittom) 157
Protein metabolism, influence of excessive water ingestion on (Orr) 530
Protein solutions, viscosity of (Chick) 261
Protein solutions, viscosity of (Chick and Lubrzynska) 59
Proteins, Herzig and Meyer's reaction applied to (Burn) 154
Proteins, of ox and horse serum, determination of the composition of, by van Slyke's method (Hartley) 541
Proteolytic organism, study of a (Drummond) 38
Pseudoglobulin, relationship of, to euglobulin in serum (Chick) 404
Pseudoglobulin, viscosity of solutions of (Chick) 261
Pseudomuscarine, constitution of (Ewins) 209
Purine metabolism of rats (Ackroyd) 434

RAPER, H. S. The gravimetric estimation of minute quantities of phosphorus 649
RAPER, H. S. The mode of oxidation of certain fatty acids with branched chains 320
Rats, purine metabolism of (Ackroyd) 434
Reductase, action of poisons on (Harris and Creighton) 585

Reductase, attempts to isolate (Harris and Creighton) 585
Reductase of washed zymin and dried yeast (Harden and Norris) 100
Regulator mixtures, notes on (Walpole) 628
Respiratory exchange in fresh-water fish (Gardner and Leetham) 374, 591
Rice-polishings, chemical investigation of the phosphotungstate precipitate from (Drummond and Funk) 598
ROSENHEIM, M. C. The cholesterol of the brain. II. The presence of "oxycholesterol" and its esters 74
ROSENHEIM, M. C. The cholesterol of the brain. III. Note on the cholesterol contents of human and animal brain 82
ROSENHEIM, O. The galactosides of the brain. II. The preparation of phrenosin and kerasin by the pyridine method 110
ROSENHEIM, O. The galactosides of the brain. III. Liquid crystals and the melting point of phrenosin 121
ROSENHEIM, O. and DRUMMOND, J. C. A volumetric method for the estimation of ethereal and inorganic sulphates in urine 143
ROTHERA, A. C. H. *see* JACKSON, L. C. 1

SARKAR, S. L. Colouring matter contained in the seed-coats of *Abrus precatorius* 281
SCHRYVER, S. B. A note on the production of casein from caseinogen 152
Seeds, urease content of certain Indian (Annett) 449
Serum, ox and horse, determination of composition of proteins of, by van Slyke's method (Hartley) 541
SKELTON, R. F. *see* PLIMMER, R. H. A. 70, 641
Sols, diagrammatic co-ordination of phenomena relating to aggregation of (Walpole) 170
Specific precipitate, behaviour of trypsin in presence of (Porter) 50
Sugar, estimation of, in blood (Gardner and MacLean) 391
Sulphates, estimation of, in urine volumetrically (Rosenheim and Drummond) 143
SUTHERLAND, W. D. and MITRA, G. C. Remarks on Dr Symons' "Note on a Modification of Teichmann's Test for Blood" 128
Symons' note on Teichmann's blood test, remarks on (Sutherland and Mitra) 128

Teichmann, remarks on Symons' note on the blood test of (Sutherland and Mitra) 128
Trout, brown, respiratory exchange in (Gardner and Leetham) 374, 591
Trypsin, action of, on pepsin (Edie) 193
Trypsin, behaviour of, in presence of a specific precipitate (Porter) 50
Trypsin, resistance of solutions of, to heat (Edie) 84
Trypsinogen, activation of (Vernon) 494
TULLOCH, W. J. The mechanism of agglutination of bacteria by specific sera 293

Urea, estimation of, in urine by urease (Plimmer and Skelton) 70
Urease, content of certain Indian seeds (Annett) 449
Urease, employed for estimation of urea and allantoin in urine (Plimmer and Skelton) 70
Urine, estimation of allantoin in, in presence of glucose (Plimmer and Skelton) 641
Urine, estimation of ethereal and inorganic sulphates in, volumetrically (Rosenheim and Drummond) 143
Urine, estimation of urea and allantoin in, by means of urease (Plimmer and Skelton) 70

Van Slyke, determination of composition of proteins of ox and horse serum by the method of (Hartley) 541
VERNON, H. M. The activation of trypsinogen 494
Viscosity of protein solutions (Chick) 261
Viscosity of protein solutions (Chick and Lubrzynska) 59
Vitamine, relations to lipoids (Cooper) 347

WALPOLE, G. S. An improved hydrogen electrode 131
WALPOLE, G. S. Diagrammatic co-ordination of phenomena relating to aggregation of sols 170
WALPOLE, G. S. Notes on regulator mixtures, recent indicators, etc. II. 628
Water, influence on protein metabolism of excessive ingestion of (Orr) 530
WHELDALE, M. and BASSETT, H. Ll. The flower pigments of *Antirrhinum majus*. III. The red and magenta pigments 204

Yeast, autolysed, curative action of, against avian polyneuritis (Cooper) 250
Yeast, dried and washed, enzymes of (Harden and Norris) 100
Yeast, dried and washed, enzymes of (Harden and Zilva) 217
Yeast, dried and washed, reductase of (Harden and Norris) 100
Yolk of egg, osmotic phenomena of (Osborne and Kincaid) 28
YOUNG, W. J. A note on the black pigment in the skin of an Australian black 460

ZILVA, S. S. *see* HARDEN, A. 217
ZILVA, S. S. The rate of inactivation by heat of peroxidase in milk. I. 656
Zymin, washed, enzymes of (Harden and Norris) 100
Zymin, washed, enzymes of (Harden and Zilva) 217
Zymin, washed, reductase of (Harden and Norris) 100

CAMBRIDGE: PRINTED BY JOHN CLAY, M.A. AT THE UNIVERSITY PRESS

Torsion Balance, for rapid weighings, calibrated from 0–500 mgrs. in 500 divisions.

MADE IN ENGLAND.

APPARATUS FOR THE MICRO-ESTIMATION OF

SUGAR IN BLOOD

BY

Dr Ivar Bang's Method.

Write for our Circular No. 168.

A. GALLENKAMP & Co., Ltd.,

Contractors to the War Office, Crown Agents for the Colonies, London County Council, etc., On the Admiralty List,

19 and 21, Sun Street, Finsbury Square, LONDON, E.C.

University of Chicago Press

ARTIFICIAL PARTHENOGENESIS AND FERTILIZATION

By Jacques Loeb, Member of the Rockefeller Institute for Medical Research.

318 pages, 12mo, cloth; 10*s.* net.

This new work presents the first complete treatment of the subject of artificial parthenogenesis in English. Professor Loeb published four years ago a book in German under the title *Die chemische Entwicklungserregung des tierischen Eies.* Mr W. O. R. King, of the University of Leeds, translated the book into English, and the translation has been revised, enlarged, and brought up to date by Professor Loeb. It gives, as the author says in the preface, an account of the various methods by which unfertilized eggs can be caused to develop by physico-chemical means, and the conclusions which can be drawn from them concerning the mechanism by which the spermatozoon induces development. Since the problem of fertilization is intimately connected with so many different problems of physiology and pathology, the bearing of the facts recorded and discussed in the book goes beyond the special problems indicated by the title.

British Medical Journal. The subject of the book is an analysis of the mechanism by which the male sex cell—the spermatozoon—causes the animal egg to develop. The author has gained a world-wide reputation for his achievements in artificial fertilization, and this work shows how, according to his observations, the action of well-known chemical and physical agencies may be substituted for that of the living spermatozoon.

The publications of the University of Chicago Press, a full catalogue of which will be sent on application, are sold in the United Kingdom and the British Colonies by THE CAMBRIDGE UNIVERSITY PRESS, Fetter Lane, London, E.C.

THE CAMBRIDGE PUBLIC HEALTH SERIES

Under the Editorship of

G. S. GRAHAM-SMITH, M.D., AND J. E. PURVIS, M.A.

Flies in Relation to Disease. Bloodsucking Flies. By EDWARD HINDLE, B.A., Ph.D., Assistant to the Professor of Biology, Cambridge. Demy 8vo. With 88 text-figures. 12*s.* 6*d. net.*

The author's main object in writing this book has been to collocate the more important observations concerning the part taken by biting flies in the transmission of disease. In doing this it seemed advisable to include notes on the classification of the flies concerned and also descriptions of the infections transmitted, but no attempt has been made to give any account of the clinical symptoms of the various diseases, whether of man or animals. Special attention has been devoted to the modes of life of the more important insects mentioned, to the manner in which the infection is transmitted from one host to another, and also to any preventive measures directed either against the flies or the infections themselves

Flies in Relation to Disease. Non-Bloodsucking Flies. By G. S. GRAHAM-SMITH, M.D., Lecturer in Hygiene in the University of Cambridge. Demy 8vo. Second edition, revised and enlarged. With 27 plates, 32 text-figures and 20 charts. 12*s.* 6*d. net.*

"The fact that the book is published at the Cambridge University Press is sufficient guarantee of the excellence of the printing and binding....We can only say that if the other volumes of this series...are as good as this one, a very high standard of excellence will have to be maintained. We consider that no one in the Public Health Service should be without this book."—*Medical Times* on the first edition

The Chemical Examination of Water, Sewage, Foods and other Substances. By J. E. PURVIS, M.A., University Lecturer in Chemistry and Physics as applied to Hygiene and Public Health, Cambridge, and T. R. HODGSON, M.A., Public Analyst for the County Boroughs of Blackpool and Wallasey. Demy 8vo. 9*s. net.*

This book is intended for the use of students who are attending courses of instruction for diplomas and degrees in Public Health, and for those who are studying the chemistry of water, sewage, sewage effluents, foods, disinfectants, etc. during their ordinary laboratory practice. It will probably be also useful to those who are engaged as public analysts or in public health matters generally. It does not give an exhaustive account of all the available methods of examination, but it describes those which have been used and tested by the authors both in laboratory instruction and in the daily work of a public analyst.

The Bacteriological Examination of Food and Water. By WILLIAM G. SAVAGE, B.Sc., M.D., D.P.H., County Medical Officer of Health, Somerset. Demy 8vo. With 16 illustrations. 7*s.* 6*d. net.*

"A hearty welcome will be accorded this compact guide, not only because it is eminently practical in its methods and directions, but also because it has been written by a man who has been accustomed to do the work himself and to depend upon it as a basis for administrative action....To all engaged in the administration of the public health...the book may be cordially commended."—*Birmingham Daily Post*

Isolation Hospitals. By H. FRANKLIN PARSONS, M.D., D.P.H., formerly First Assistant Medical Officer of the Local Government Board. Demy 8vo. With 55 text-figures. 12*s.* 6*d. net.*

"Eminently practical in character, it is at the same time complete in its information, so that the medical officer may rely with confidence on finding accurate and impartial facts on matters connected with the isolation of infectious cases....The volume should appeal very strongly to all medical officers of health, tuberculosis officers, and sanitary authorities, to whom it can be recommended with all heartiness."—*Aberdeen Free Press*

A list of volumes in preparation will be sent on application

CAMBRIDGE UNIVERSITY PRESS, FETTER LANE, LONDON
C. F. CLAY, Manager

THE JOURNAL OF BIOLOGICAL CHEMISTRY

A Monthly Publication devoted to original Chemical Investigations in the Biological Sciences

Each volume contains between five and six hundred pages.

The subscription price per volume is $3.00 in the United States and Canada; in other countries $3.25.

Remittances should be made payable to *The Journal of Biological Chemistry* and sent to 2419—21 York Road, Baltimore, Maryland, U.S.A. or to Dr A. N. Richards, University of Pennsylvania, Philadelphia, Pa., U.S.A.

Announcement is made of the following offers, made possible by the existence of the Christian A. Herter Memorial Fund.

1. Individuals known to be engaged in biochemical work who subscribe to the Journal at the usual rates beginning with Volume XIV (now current), may secure Volumes I—XIII for the sum of $20.00, plus cost of transportation. The price at which a complete set has hitherto been sold is $50.00. Applications for these volumes under the terms of this offer should be sent directly to the Secretary, Journal of Biological Chemistry, Philadelphia, Pa., and should be accompanied by such information concerning the work and occupation of the applicant as will enable the Directors to decide whether he may properly be included within the intention of the offer.

2. Individuals or Institutions who are already subscribers to the Journal and who wish to complete their files may secure early volumes for $1.50 each, plus cost of transportation, while the supply of back numbers reserved for this purpose remains unexhausted.

The Directors of the Journal specifically reserve the right to carry out the distribution of the Journal under the terms of these offers within their discretion and to withdraw the offers without notice when the limited number of volumes available shall be exhausted.

The Annals of Applied Biology

THE OFFICIAL ORGAN OF THE ASSOCIATION OF ECONOMIC BIOLOGISTS. Edited by Professor MAXWELL LEFROY, Imperial College of Science and Technology, London.

The Association of Economic Biologists commences under the above title a publication devoted mainly to the scientific papers read by members at the meetings and devoted to those branches of Biology in which the Association has been interested. During the ten years of the Association's life, its meetings have discussed mainly horticultural, agricultural and forest biology; the specialised parts of agricultural science, of genetics and of medical zoology are dealt with in other journals and the *Annals* will appeal more to those interested in the diseases and pests of plants, scientific problems of horticulture and forestry, tropical economic botany and agricultural zoology in the stricter sense.

The *Annals* will be edited by an Editorial Committee, including a General Editor and Editors for each branch of Applied Biology in which members are interested. The scope of the *Annals* is as wide as that of the membership of the Association, and all members are invited to send in papers and notes to the Secretary of the Association.

Terms of Subscription.

Members of the Association will receive the *Annals* free. To others the annual subscription price, including postage to any part of the world, for a single copy of each of the four parts making up the annual volume, is 25*s.* net; single copies 7*s.* 6*d.* net each. Subscriptions for the *Annals* are payable in advance and should be sent to Mr C. F. CLAY, Cambridge University Press, Fetter Lane, London, E.C., either direct or through any bookseller.

The publishers have appointed the University of Chicago Press agents for the sale of the *Annals of Applied Biology* in the United States of America and have authorised them to charge the following prices: annual subscription post free, $6, single copies, $2.

CONTENTS OF VOLUME VIII, No. 6, DECEMBER 1914.

(*All rights reserved*)

PAGE

LVIII. The Action of Poisons on Reductase and Attempts to isolate this Enzyme. By DAVID FRASER HARRIS and HENRY JERMAIN MAUDE CREIGHTON 585

LIX. On the Respiratory Exchange in Fresh-Water Fish. Part II: On Brown Trout. By JOHN ADDYMAN GARDNER and CONSTANCE LEETHAM. (With 1 Text-figure) 591

LX. The Chemical Investigation of the Phosphotungstate Precipitate from Rice-Polishings. By JACK CECIL DRUMMOND and CASIMIR FUNK . 598

LXI. A Comparison between the Molecular Weights of Protagon and of the Phosphatide and Cerebrosides obtainable from it. By ALEXANDER LYALL PEARSON 616

LXII. Notes on Regulator Mixtures, Recent Indicators, etc. II. By GEORGE STANLEY WALPOLE. (With 1 Text-figure) 628

LXIII. The Estimation of Allantoin in Urine in the Presence of Glucose. By ROBERT HENRY ADERS PLIMMER and RUTH FILBY SKELTON 641

LXIV. The Gravimetric Estimation of Minute Quantities of Phosphorus. By HENRY STANLEY RAPER 649

LXV. The Rate of Inactivation by Heat of Peroxidase in Milk. I. By SYLVESTER SOLOMON ZILVA. (With 4 Text-figures) 656

LXVI. Liver Nitrogen in Anaphylaxis. By GEORGE BARGER and HENRY HALLETT DALE 670

For Notice to Contributors see page 2 of Wrapper

It is hoped that the next number will appear about the end of March. Contributions intended for publication in it must be in the hands of the Editors before February 10th.

C 37
0585
1 32E
2 5E3
3 32E
4 283
5 582
6 E38